

## **Review on Different Diagnostic Methods for Detection of *Mycobacterium avium* Subsp. *Paratuberculosis* infections**

<sup>1</sup>Hailu Dugassa and <sup>2</sup>Tilaye Demisie

<sup>1</sup>Institute of Tropical Medicine Hailu Dugassa Girsha Sint-Rochustraart 38,  
203 2000 Antwerp Belgium

<sup>2</sup>Faculty of Veterinary Medicine Addis Ababa University P. O. Box 38 Debrezeit Ethiopia

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**Abstract:** *Mycobacterium avium subsp paratuberculosis* (MAP) is a veterinary pathogen and unambiguously causes John's disease or Paratuberculosis in ruminants and wild species including primates. The diagnosis of paratuberculosis has not been improved significantly and still relies on the acid fast staining and culture of clinical specimen which are developed before several decade years. ZN cannot differentiate among acid fast microorganisms and antigens shared by different mycobacteria infections and Map detection by means of bacterial culture in solid medium is still the reference diagnostic method, but it requires 6 to 8 months to complete. However, it is slow and insensitive, especially at the early stages of infections. 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. Fortunately, new diagnosis technologies are in continuing development and much hope has been laid on the development of different diagnostic approaches for rapid detection of *Mycobacterium avium subsp. Paratuberculosis* infections. Different diagnostic methods such as: Pathological diagnoses, Johnin tuberculin test, Immunohistochemistry, In situ hybridization and In Situ polymerase chain reaction have been appeared in the field of Paratuberculosis. The principles, methods and applications of different diagnostic methods for detection of *Mycobacterium avium subsp. Paratuberculosis* infections have been discussed. The advantages and disadvantages of using these diagnostic methods for detection of *Mycobacterium avium subsp. Paratuberculosis* infections have been elegantly shown. Comparison of different diagnostic approaches for diagnosis of Paratuberculosis was discussed. ISH and direct in situ PCR should be considered as the methods choice for detection of spheroplasts (forms of MAP with deficiencies in the cell wall) which may be not detected by ZN or IHC in formalin fixed paraffin embedded tissue samples. But, IHC is considered as the method of choice for detection of Map in formalin fixed and paraffin embedded tissue samples. IHC would give better results since antigen retrieval process can overcome some detrimental effects of fixation. Further studies should be carried out to evaluate sensitivity, specificity and alternative protocols for IHC, ISH and ISP techniques.

**Key words:** Diagnostic Methods • Detection • *Mycobacterium avium subsp. paratuberculosis* • Infections • Paratuberculosis

### **Abbreviations:**

Ab: Antibody  
AFB: Acid Fast Bacilli  
AFOs: Acid Fast Organisms  
Ag: Antigen  
CD: Crohn's Disease  
CFU: Colony Forming Unit  
CMI: Cell Mediated Immunity

CWD: Cell Wall Deficient  
dISP: direct In Situ Polymerase Chain Reaction  
FISH: Fluorescence labeled In Situ Hybridization  
H and E: Hematoxlin and Eosin  
IHC: Immunohistochemistry  
ISH: In Situ Hybridization  
ISP: In Situ Polymerase Chain Reaction  
JD: Johnne's disease  
MAP: *Mycobacterium avium subsp paratuberculosis*  
PCR: Polymerase Chain Reaction  
PI-PCR: Prokaryotic In Situ Polymerase Chain Reaction  
PPD: Purified Protein Derivatives  
PTB: Paratuberculosis  
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction  
ZN: Ziehl Neelsen staining

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## INTRODUCTION

Paratuberculosis (PTB) or Johnne's disease is a chronic infectious disorder caused by an acid fast (AF) bacillus, *Mycobacterium avium subsp. paratuberculosis* (Map). The disease affects cattle and other animal species and is characterized by diarrhea, weight loss and eventually death [1, 2]. In infected animals, Map causes granulomatous enteritis and lymphadenitis with mononuclear infiltration of macrophages, lymphocytes, plasmatic and epithelioid cells. Langhans-type giant cells and necrotic areas could also be observed [2]. Since PTB shares many Characteristics with Crohn's disease, a relationship between both diseases has been postulated and Map and its cell wall-deficient forms called spheroplasts have been associated with human disease [3]. MAP has wide host range and able to survive outside host for long time (More than 9 months), so it seem more insidious than any other bacteria [3].

*Mycobacterium avium* subspecies *paratuberculosis* (*M. paratuberculosis*) is extremely slow growing, mycobactin-dependent and highly resistant to the host immune response [4]. Therefore, infected animals may harbor the organism for years before they develop the disease. The disease occurs worldwide with enormous economic losses [5]. Its control or eradication is limited by the lack of sensitive diagnostic tests to identify and eliminate infected animals at the subclinical stage of the disease [6]. Although primarily considered as the cause of a ruminant disease, the cell wall deficient (CWD) form of this or a closely related organism has also been implicated as a possible cause of idiopathic diseases with histologic features similar to JD such as sarcoidosis and Crohn's disease (CD) in humans [7].

Histopathology is used as a diagnostic method, but it is also a very important tool for researching PTB. Detection of Map in tissue samples improves the pathologic diagnosis and may be necessary when experimental infections are performed. Several techniques such as Ziehl Nielsen staining (ZN), immunohistochemistry (IHC) and in situ hybridization (ISH) were tested for detection of the agent [8], but their performances are different. ZN and IHC are easy to perform and have high sensitivity [8, 9], but false negative can arise when infection was recent or bacilli were scanty. Besides, their specificity may be considered low since ZN cannot differentiate among acid fast microorganisms and antigens shared by different mycobacteria may affect IHC performance. When both are compared, ZN is cheaper, but IHC may detect antigens of Map even when the bacillus was digested in the cytoplasm of macrophages. On the other hand, ISH is specific but more expensive, hard to perform and its interpretation may be difficult because of the lower signal obtained [8]. However, detection of Map by ISH is considered useful because it detects spheroplasts (Forms of Map with deficiencies in the cell wall) which may be involved with the disease and may be not detected by ZN or IHC [10]. Besides, detection of DNA of Map may be useful when IHC and ZN are negative because bacilli and their antigens are damaged. The information about IHC spheroplast detection is scanty but ISH and direct in situ PCR (dISP) are considered useful for the detection of cell wall-deficient forms of Map in tissues samples [11].

In situ PCR (ISP) consists of the amplification of one specific sequence of DNA in a tissue sample. It has been described as a very sensitive and specific technique and is used for diagnosis or research in many diseases.

Related to PTB, an ISP method followed by in situ hybridization was performed for detection of Map in sheep and mice tissue samples [12, 13]. Although it was useful for Map identification, a direct ISP (dISP) method which does not require the hybridization step should be easier to perform. Detection of Map in tissue samples supports pathological diagnosis and allows to detect where the agent persists in the suspected lesions of paratuberculosis [13, 14]. The dISP was able to detect Map in all formalin-fixed, paraffin-embedded tissue samples from naturally infected cattle [11]. It was described that spheroplasts may be related to the development of PTB or CD and these forms of Map can be detected by ISH [10, 12]. In a similar way, dISP would be useful for their identification since it is based in the detection of mycobacterial DNA and this fact may improve its sensitivity when compared to IHC [11].

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 [15]. Thus, up to date and reliable information is needed to choose more accurate diagnostic methods for detection of *Mycobacterium avium subsp. paratuberculosis* infections.

Therefore, the objectives of this review paper are:

- To review the most widely used diagnostic methods for detection of *Mycobacterium avium subsp. paratuberculosis* infections.
- To discuss the advantage and disadvantage of each of these diagnostic methods for detection of *Mycobacterium avium subsp. paratuberculosis* infections.

### **Pathological Diagnosis of Paratuberculosis**

**Morphological (Gross) Lesions:** Different research findings have indicated that many infected animals do not have gross lesions, there is not always a close correlation between the severity of clinical signs and the extent of intestinal lesions and a wide range of specimens must be examined to ensure a reliable diagnosis. In cattle, small ruminants and deer the gross pathological findings are similar. Necrosis rarely occurs in cattle and, unlike sheep, goats and deer, there is no caseation or calcification [16]. Clinically affected animals are usually emaciated, have serous atrophy of fat and effusion in the body cavities. The primary macroscopic lesions of Johns disease in

ruminants are usually confined to the ileum, caecum, colon and draining lymph nodes [17]. The earliest lesions are thickening and cording of mesenteric lymphatics. In sheep, goats, deer and alpaca the lymphatics may have small, white miliary nodules of caseous necrosis along their length [18].

The mesenteric and ileocaecal lymph nodes are always enlarged, oedematous and may have focal or diffuse pallor in the cortex. In some goats, sheep and deer, areas of caseous necrosis and mineralization are present in the cortex and appear as white foci 1-4 mm in diameter [18, 19]. The enteric lesions are most common in the terminal ileum and vary from mild, velvety thickening of the mucosa to severe thickening of the bowel with transverse corrugation of the mucosal surface. Necrotic foci in the intestinal mucosa may be found in goats, sheep and deer [16]. In addition, mucosal hyperaemia, erosions and petechiation have been observed in deer. Tubercle-like lesions have also been observed in lymph nodes of the head in deer. Gross changes in deer are very difficult to distinguish from lesions caused by *M. bovis* or other members of the *M. avium* complex [18].

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 [2]. 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 [20].

Paratuberculosis 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. Focal lesions are supposed to be the first to appear and are associated with a strong cellular immune



Fig. 1: Gross pathology of an ileum section from apparently healthy sheep. Corrugations and thickening of the mucosal wall are prominent [21].

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 hypoproteinemia and edema. The lepromatous type appears in some animals, related to the changing profile of the immune response [2].

**Histopathological Diagnosis:** Histological lesions of Johns disease are characterized by the presence of aggregations of large macrophages with abundant granular cytoplasm, often referred to as epithelioid cells, in the intestinal mucosa and submucosa, lymphatics and in the cortex of mesenteric lymph nodes [22]. In the intestines, these aggregations of macrophages are accompanied by focal or diffuse infiltration composed of lymphocytes, eosinophils and occasional neutrophils [23]. Multinucleate giant cells are seen in the intestinal mucosa and cortex of the mesenteric lymph nodes of cattle, deer and small ruminants [22].

In some cases, there are focal aggregates of macrophages and scanty acid-fast organisms (AFOs) in the lamina propria. This type of granulomatous inflammatory reaction is frequently classified as paucibacillary, tuberculoid, whereas the diffuse infiltration of the intestinal mucosa and submucosa with macrophages that are laden with numerous AFOs is referred to as a multibacillary, lepromatous reaction [23]. The diffuse infiltration of the intestinal mucosa is associated with atrophy of villi and a decrease in the number of crypts [19].

In some studies, focal areas of caseation and calcification have been observed in the bowel and mesenteric lymph nodes of sheep and goats [24]. However, other workers have either failed to identify such lesions, or have attributed them to parasitic infestation.

Extensive fibrosis and necrosis in the mesenteric lymph nodes and, in some cases, in lymph nodes of the head is a feature of paratuberculosis in deer [25]. Identification of the organism by bacterial culture or PCR is required to distinguish lesions in mesenteric lymph nodes and lymph nodes of the head caused by *M. paratuberculosis* from those caused by *M. bovis* and *M. avium* [25].

Gross lesions in the liver have rarely been reported in sheep [17], but microgranulomas may be scattered throughout the hepatic parenchyma [19]. In cattle, aggregates of globule leukocytes have been observed in, or around, myenteric ganglion cells. Lymphocytic neuritis in the gut has been reported in sheep [26]. In all ruminant species, AFOs are usually numerous within epithelioid cells and multinucleate giant cells in intestinal sections. Fewer AFOs are present in the mesenteric lymph nodes and they are scanty in liver lesions [19]. Optical microscope observation after ZN shows  $1.5 \times 0.5 \mu$  acid-fast bacilli, in clumps or within macrophages [27].

A diagnosis of lesions consistent with *M. paratuberculosis* infection is indicated if in any one section, one or more single giant cells and/or one or more accumulations of three epithelioid macrophages are observed in the intestinal lamina propria and/or lymph node cortex with the presence of at least one acid-fast bacillus (In New Zealand the sighting of three acid fast bacilli is required) morphologically consistent with *M. paratuberculosis*. A finding suggestive of *M. paratuberculosis* infection is indicated if in any one section, two single Langhans giant cells and/or two accumulations of three epithelioid macrophages in the intestinal lamina propria and/or lymph node cortex are observed without the detection of an acid-fast bacillus [28].

#### **Advantage and Disadvantage of Pathological Diagnosis:**

The advantage of the pathological diagnosis is that it allows identifying animals with focal lesions associated with subclinical stages, in which 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 [29]. Whitlock and Buergelt (1996) [30] 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.

#### **Bacteriological Examination for Detection of *Mycobacterium Avium Subsp. Paratuberculosis* Infections**

**Ziehl-Neelsen (ZN) Staining:** The bacterioscopic method

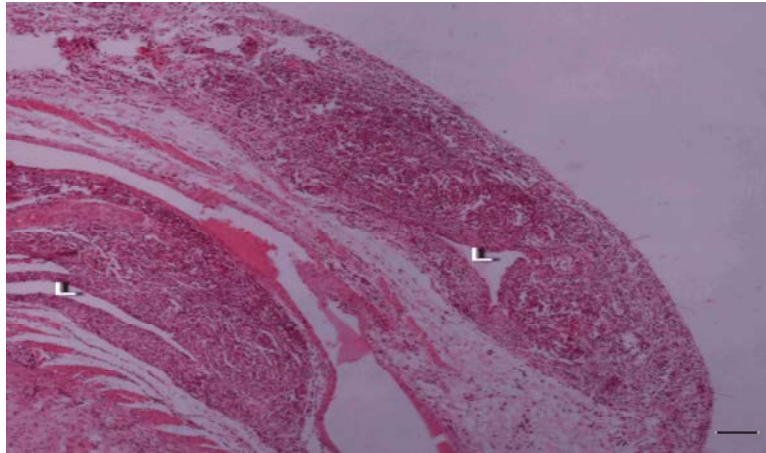


Fig. 2: Histopathological section of the ileum (Muscularis and serosa) from apparently healthy/subclinically infected sheep. Granulomatous lymphangitis is shown where mononuclear cells infiltrate the wall of the lymphatics. H&E stain, L = lymphatic vessels, bar = 100 μ [21].

Table 1: The description of the stages of bovine PTB related to clinical signs and likelihood of diagnosis [31].

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 Submamaxilar edema, cachexia persistent diarrhea	+	+

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 [31]. Examination of Ziehl-Neelsen stained faecal smears can be applied in clinical cases to demonstrate typical clumps of acid-fast bacilli. However, false negative results occur and while the presence of acid-fast bacilli in clumps of three or more cells morphologically consistent with *M. paratuberculosis* is consistent with John's disease; this is not a definitive test [28].

A positive control (Smear containing/spiked with *M. paratuberculosis*) must be included in each batch of specimens for microscopy. Acid-fast bacteria stain red. Other organisms and organic material stain green or blue depending on counterstain [28]. In smears or sections of tissues, 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 (Table 1) [31].

**Advantage and Disadvantage of Ziehl-Neelsen Staining:**

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 [31].

**Bacterial Culture:** The isolation of *M. paratuberculosis* from faeces or tissue is the definitive test for diagnosis of John's disease. There are several culture methods, which vary with respect to media, sample type and sample

processing protocols. The cultivation of *M. paratuberculosis* is always performed using special media supplemented with mycobactin J [28]. Since *M. paratuberculosis* organisms are vastly outnumbered by other bacteria or fungi in faecal and intestinal tissue specimens, the successful isolation of the target organism depends on efficient inactivation of the undesirable microbes. However, the decontamination process has a negative effect on *M. paratuberculosis*. Routine decontamination protocols decrease the number of *M. paratuberculosis* organisms by about 2.7 log<sub>10</sub> and 3.1 log<sub>10</sub> for faeces and tissues, respectively. Hexadecylpyridinium chloride (HPC) is recommended as the decontaminant of choice [32].

There are two techniques for the isolation of *M. paratuberculosis*: (i) conventional culture on solid media and (ii) liquid media culture. The latter method reduces the time required for obtaining a result and is considered to be the more sensitive technique [33]. The decontamination protocol involving double incubation of faecal samples in HPC and a mixture of antibiotics (Vancomycin, amphotericin and nalidixic acid) may further improve culture sensitivity. The addition of ampicillin to the media has been reported to reduce the growth of undesired microbes [34].

Primary colonies of *M. paratuberculosis* on solid media may be expected to appear any time from 5 weeks to 6 months after inoculation. The sheep strains grow less well than the cattle strains and primary cultures on solid media should not be discarded as negative without prolonged incubation, for up to 6-8 months [35].

Colonies of the cattle strain of *M. paratuberculosis* on HEY medium are typically convex, off-white to cream or buff coloured and non-chromogenic. As cultures age and media dry out, colonies and media tend to become more buff- or beige-coloured and more raised. Colonies are soft, moist, glistening, non-mucoid and remain miscible with water [35]. Colony size is initially pin point and many remain at 0.25 to 1 mm and tend to remain small when colonies are numerous on a slope. Older isolated colonies may reach 2 mm. On modified 7H10 medium, colonies of the cattle strain are less convex than those on HEY, especially in aged cultures; are pinpoint to approx 1 mm in diameter and being buff-coloured are only slightly lighter than the media. Compared with colonies of cattle strains on HEY, those on 7H10 are more difficult to detect (Due to less contrast in colour between colony and medium) and to differentiate from colonies of some other mycobacteria [28, 35].

Colonies of the sheep strain of *M. paratuberculosis* on modified 7H10 are convex, soft, moist, glistening, off-white to buff and very similar to the colour of the media. Colonies are typically between pinpoint and 0.5 mm, but can reach 1 mm and rarely 1.5 mm if few colonies occur on a slope. Saprophytic mycobacteria may have a similar appearance on either medium but are often evident after 5-7 days. Other organisms may grow on both media with colonies appearing after days or months [35].

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 [36]. In order to reduce costs, the fecal culture can be made in groups of 3-5 individual samples, without losing too much sensitivity [37]. 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 [38]. Since infected cattle can excrete 108-12CFU/g of feces and contaminate the environment, culturing pasture soil samples from manure and/or delivery areas is also recommended [39, 40].

#### **Advantage and Disadvantage of Bacteria Culture:**

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 [15].

**Johnin Skin Tuberculin Test:** The skin test for delayed-type hypersensitivity (DTH) measures CMI, but has limited value. The test is still required by some overseas countries for certification of imported cattle and sheep [28]. The test is conducted in the same manner as the tuberculin test using johnin PPD or *M. avium* PPD. The test is conducted by intradermal inoculation of 0.1 ml johnin PPD into the caudal fold or the mid-neck region. The skin thickness is measured with calipers before and 72 hours after inoculation, an increase in skin thickness of >3 mm being taken as positive [28].

In a recent study in which Johnin (ID-Lelystad, The Netherlands) was used to test cattle, the skin test specificity was 88.8% at the cut-off value of  $\geq 2$  mm, 91.3% at the cut-off value of  $\geq 3$  mm and 93.5% at the cut-off



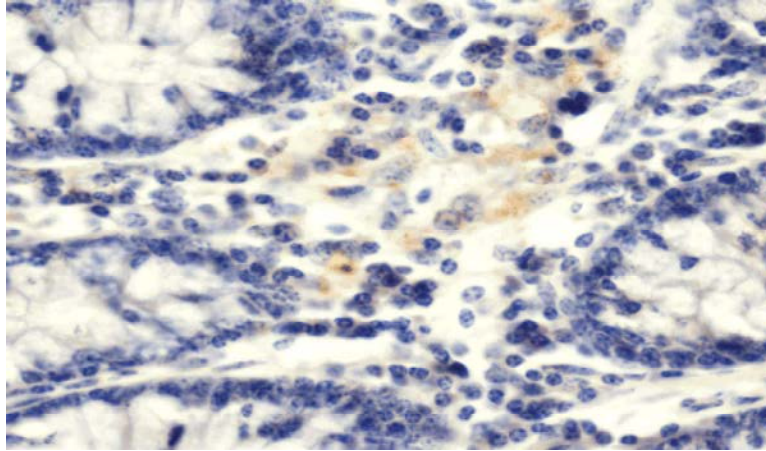


Fig. 3: Positive immunostaining (Brown color) is indicated inside the cytoplasm of epithelioid cells. Tissue's architecture is perfectly conserved (Lamina propria, ileum) [11].

value of  $\geq 4$  mm. The effect of these cut-off values on the sensitivity has not been determined. The performance of this test may also be significantly affected by minor antigenic differences that occur in different batches of antigen [41].

#### **Advantage and Disadvantage of Johnin Tuberculin Test:**

It should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitization to the *M. avium* complex is widespread in animals and neither avian tuberculin nor Johnin are highly specific [42]. Furthermore, the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria [42].

#### **Immunohistochemistry (IHC) for Detection of *Mycobacterium Avium Subsp. Paratuberculosis* Infections**

**Principle of Immunohistochemistry:** Immunohistochemistry (IHC) combines anatomical, immunological and biochemical techniques to identify discrete tissue components by the interaction of target antigens with specific antibodies tagged with a visible label. IHC makes it possible to visualize the distribution and localization of specific cellular components within cells and in the proper tissue context. While there are multiple approaches and permutations in IHC methodology, all of the steps involved are separated into two groups: sample preparation and labeling [43].

Coons *et al.* [43] describes an immunofluorescence technique for detecting cellular antigens in tissue sections marked the beginning of immunohistochemistry (IHC). Since then, IHC has become a valuable tool in both diagnosis and research of infectious and neoplastic diseases in a variety of animals. The basis of IHC is very simple and bridges three scientific disciplines: immunology, histology and chemistry. The fundamental concept behind IHC is the demonstration of antigens (Ag) within tissue sections by means of specific antibodies (Abs). Once antigen-antibody (Ag-Ab) binding occurs, it is demonstrated with a colored histochemical reaction visible by light microscopy or fluorochromes with ultraviolet light. Although conceptually simple, the methodology of IHC has become more complex as more stringent goals of sensitivity and specificity are established. Initially, simple (Direct) methods were used that produced quick results but lacked sensitivity. Currently, extremely sensitive methods are available to detect one or multiple Ags simultaneously or even to examine hundreds of tissues in the same section for the presence of a particular Ag (Microarray technology). Another critical advance in the 1990s was techniques to retrieve Ags that had been altered by fixation by means of heat, increasing exponentially the number of Ags detectable in routinely fixed tissues. Veterinary pathologists face many challenges when performing IHC because of the diversity of species studied and no guarantees that Abs will cross-react among different species. IHC has been established as a solid and reliable methodology for both routine diagnostic and research activities in veterinary medicine [44].

**Methods of Immunohistochemistry:** Samples of ileum or ileocaecal lymph node are used. Tissues are fixed in 10% formalin solution and embedded in paraffin following the standard histological procedures [1]. Immunohistochemistry was performed following procedures previously described [8]. Briefly, 2  $\mu$ m sections are obtained, mounted on positively charged slides and deparaffinized. Endogenous peroxidase activity is blocked with 10% hydrogen peroxide in methanol (20min) and antigenic recovery is performed by humid heat treatment (121°C, 15min) in citrate buffer (Monohydrate citrate, 10mM, pH 6). After cooling, slides are immersed in TBS buffer (50mM Tris-HCl, 300mMNaCl pH 7.6) for 20min. A blocking step is performed (BSA (Promega) 2% in TBS, 5min), after which 40mL of the anti-Map antibody (rabbit polyclonal, Queen's University Belfast, Northern Ireland, UK) diluted 1/100 in TBS is added and incubated at 4°C overnight. The reaction is revealed using the LSAB2R system (Dako Citomation System) and DAB. Slides are counterstained with Mayer Hematoxylin and coverslipped with synthetic medium [8].

Immunohistochemistry has been used to detect mycobacterial antigens in tissue sections and some authors have found that this technique has better sensitivity than molecular methods in addition to being easier to perform in resource poor settings [45]. However, with immunohistochemistry there is cross-reactivity between the different mycobacteria species [46]. All infected tissues showed immunostaining in areas with granulomatous inflammation. Staining was inside the epithelioid and Langhans giant cells, which were distributed in the ileal mucosa and submucosa [11].

**Advantage and Disadvantage of Immunohistochemistry:** This technique uses a MAP-specific antibody (Anti map Ab) marked with enzymes, which allows to visualize the reaction on the enzymatic substrate [47]. The advantage of this method is that it enables to identify spheroplasts and Map in tissue [48]. 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 anti-Map Ab used [49] and the sensitivity is low as compared with bacterial culture [9].

### **In Situ Hybridization (ISH) for Detection of *Mycobacterium Avium Subsp. Paratuberculosis* Infections**

**Principle of in Situ Hybridization:** 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 detecting 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 [10] and unicellular parasites where Map can grow [50].

In situ hybridization (ISH) is a technique that allows for precise localization of specific segment of nucleic acid within histological sections. Successful utilization of the technique requires a basic knowledge of molecular biology in combination with an ability to appreciate subtle histomorphologic changes. As such; it represents a perfect synergy between fundamental molecular biology and traditional histopathologic interpretation and is perfectly suited for veterinary pathologists [16].

In situ hybridisation (ISH), where labelled probes are used to detect complementary DNA or RNA in tissue sections or smears and [51] examination by Southern or other blotting techniques of nucleic acids extracted from homogenized biopsy material. ISH enjoys better rapport with histopathologists because of its similarity to immunohistochemistry. ISH has the unique advantage over other molecular biology techniques of allowing localization and visualization of target nucleic acid sequences within morphologically identifiable cells or cellular structures in a heterogeneous cell population. Such specific localization has a marked advantage over older molecular methods where detection of the nucleic acid target from a tissue homogenate precludes identification of the actual affected cell, appreciation of cellular detail and relations and subsequent reconstructive interpretation of the findings [51].

**Methods of in Situ Hybridization:** Tissues are fixed in 10% formalin solution and embedded in paraffin following the standard histological technique [1]. A 284-bp fragment of IS900, obtained by PCR with primers p89 and P92 as previously described [10], is used as a probe for hybridization. The probe is diluted in hybridization buffer [SSC 4\_x (1\_x SSC = 0.14 Msodiumchloride, 0.014Msodiumcitrate), Sodium Phosphate 0.2 M, Denhart



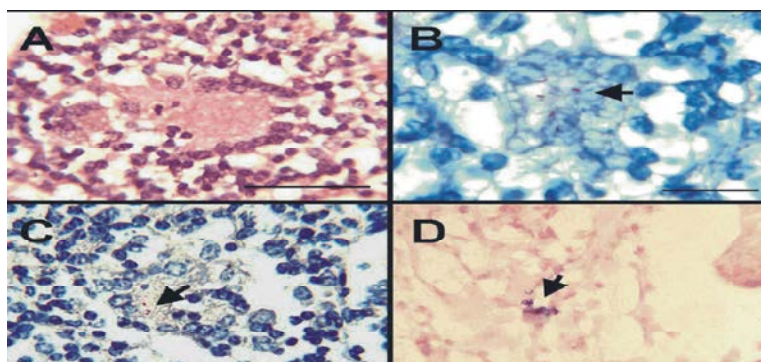


Fig. 4: An image corresponding to Ileum (A) A Langhans-type giant cell is observed, accompanied by an infiltrate of mononuclear cells as macrophages and lymphocytes. H&E. Bar, 100 mm. (B) Presence of AF bacilli inside a Langhans-type giant cell cytoplasm (Arrow), Ziehl Neelsen Bar, 50 mm. (C) Immunostaining inside a Langhans-type giant cell (Arrow). IHC. Bar 100 mm. (D) Hybridization within macrophages (Arrow). ISH. Bar, 100 mm [8].

Solution2x\_] and an equal volume of 20% sulphate dextran in formamide at 2 ng/micro l. It is denatured in boiling water for 5 min and then placed in ice. Tissue sections (1 microm) on positively charged slides are deparaffinized and incubated with Proteinase K (S3020, Ready to use, Dako1) at 37°C for 20 min. Then, they are immersed in distilled water, dehydrated and air-dry. Probe (100 micro l) was applied on the tissue surface. Slides are coverslipped and heated at 95°C (10 min). Hybridization is performed at 42°C, overnight, in a humid chamber. Washes with SSC 2x\_, 1x\_ and 0.2x\_ are carried out (15min each) at room temperature (RT) and samples are incubated with blocking solution (100mM TrisHCl; 150mM NaCl, pH 7.8, BSA (Promega) at 50 mg/ml), 15 min, at RT in a humid chamber. Alkaline phosphatase streptavidin conjugate (40 micro g/ml) diluted in conjugated buffer (100mM Tris-HCl, 150 mM MgCl<sub>2</sub>, 1% BSA pH 7.5) is added (15 min, RT, in a humid chamber). Two washes with TBS (15 min, RT) and one with substrate alkaline buffer (100mMTrisbase, 150 mMNaCl, 50mMMgCl<sub>2</sub>, pH9, 5 min, RT) are performed after which the slide is covered with 6 ml of Nitro-Blue Tetrazolium Chloride (NBT, 75mg/ml in 70% dimethylformamide) and 3.2 ml of 5-bromo-4-chloro-3-ndolylphosphate p-toluidine salt (BCIP, 50mg/ml in 100% dimethylform- amide) in 1 ml of substrate alkaline buffer (2 h, 37°C in a humid chamber). Finally, slides are counterstained with Nuclear Fast Red (Vector1 Cat. No H-3403), dehydrated and mounted with synthetic medium [8].

Hybridization was observed in samples originated in infected animals and it was localized exclusively in areas with lesions, within cytoplasm of macrophages and epithelioid cells. In ileum samples, it was also found around glandules and in the lymphatic duct lumen (Figure 4) [8].

#### Advantage and Disadvantage of in Situ Hybridization:

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 [15].

#### In situ PCR (ISP) Method for Detection of *Mycobacterium Avium Subsp. Paratuberculosis* Infections:

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence [52].

**Principle of in situ PCR:** Nearly ten years ago, a technique is called in situ PCR was successfully used to amplify and detect viral DNA inside eukaryotic cells [53]. Since then in situ PCR methods have been widely used in medical research and undergo rapid development and modification. In situ PCR has been used to specifically amplify and detect single copy or low copy of nucleic acids sequences in single cells and tissues [54]. Hodson *et al.* [55] developed a prokaryotic in situ PCR (PI-PCR) method to visualize the micro scale distribution of specific genes and gene products in individual bacterial cells in microbial communities.

PI-PCR differs from eukaryotic in situ PCR in many aspects: (1) prokaryotic cells are usually too small (Less than a few micrometers) for in situ PCR/RT-PCR to localize genes and their transcripts in intact cells under epifluorescence microscopy; (2) prokaryotic mRNAs usually have shorter half-lives (Less than 10 minutes), therefore, rapid fixation and optimal preservation are

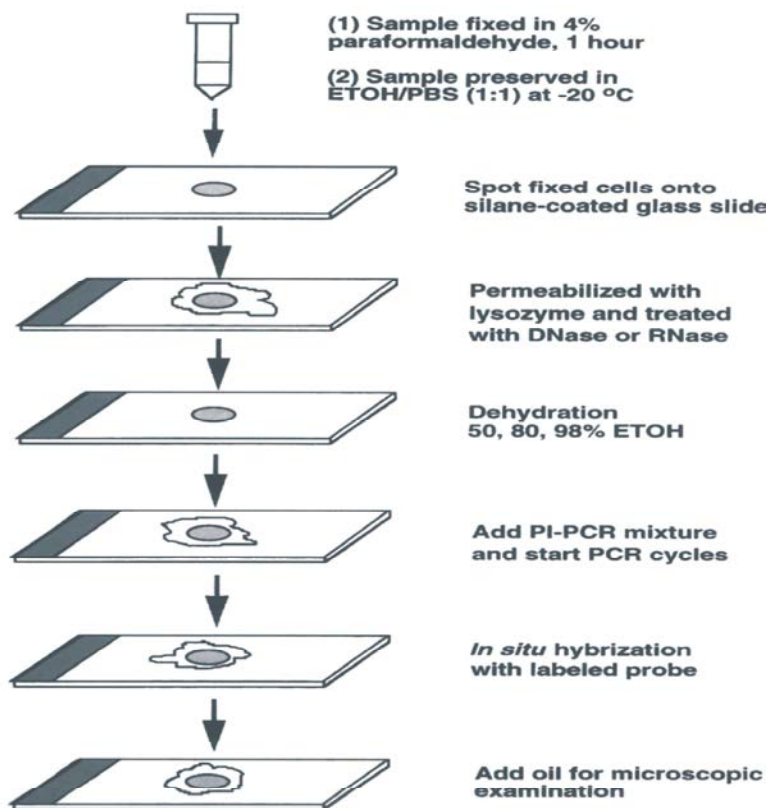


Fig. 5: The schematic presentations of the major steps in direct PI-PCR [57]

necessary to detect mRNAs transcripts using PI-RT-PCR; (3) cell wall permeabilization by lysozyme is critical for successfully PI-PCR amplification. Unlike *in vitro* PCR/RT-PCR in which extracted DNA or RNA is used as a template for amplification, PI-PCR is performed using DNA or RNA inside bacterial cells where the cell membrane acts as a container for amplified products [56].

The nature of the cellular material containing the target sequences to be amplified is an important variable for PI-PCR. Successful *in situ* PCR amplification depends on extensive optimization procedures to determine the conditions which will permeabilize the bacterial cell membranes to allow the entry of reagents for amplification and detection, yet retard the diffusions of PCR product out of the cells. If amplification target is DNA, cellular RNA should be digested with DNase-free RNase. However, if amplification target is RNA (rRNA and mRNA), permeabilized cells are treated with RNase-free DNase. Currently, there are two different methods for detecting amplified PCR products inside cells, direct or indirect *in situ* PCR. For direct PI-PCR, the fluorochrome or digoxigenin-labeled nucleotides into amplicons of prokaryotic cells during the PCR process [55]. For indirect PI-PCR, the amplified PCR product inside the prokaryotic

cells is detected by hybridization to the fluorescently labeled probe which is specific for an internal region of the PCR amplicon [56].

The basic procedure of PI-PCR/FISH involves cell fixation, cell wall permeabilization, DNase or RNase treatment, cell dehydration, *in situ* PCR amplifications and signal detection by FISH (Figure 5) [56].

**Methods of *in situ* PCR:** Tissues are fixed in 10% formalin solution and embedded in paraffin following the standard histological technique [1]. Tissue sections (2  $\mu$ m thickness) are obtained and mounted on positive charged slides. These are deparaffinised by keeping 18 h at 60°C and immersed in xylene (30min at 37°C), absolute ethanol at room temperature (RT), 75% ethanol (RT), 50% ethanol (RT), 25% ethanol (RT) and water (RT). Then, they are made permeable by incubation at room temperature in 0.02mol/L HCl for 10min, followed by 0.01% triton X-100 for 90 s. Proteins are depleted by incubation with 1mg/L proteinase K (Gibco, Paisley, UK) for 30min at 37°C, which is inactivated by boiling in microwave for 15 s. Endogenous alkaline phosphatase is inactivated by immediately immersing the slides into 20% acetic acid for 15 s [11].

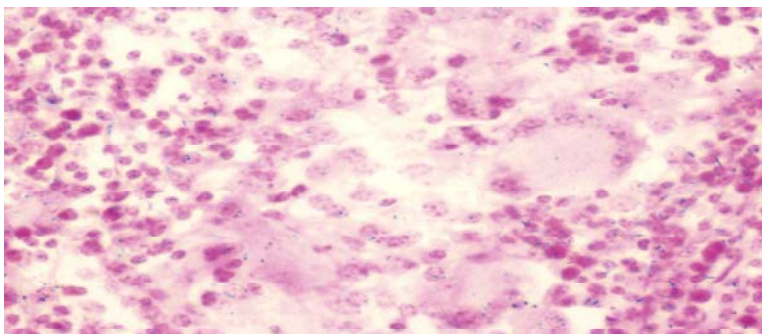


Fig. 6: Positive in situ PCR signal (Small blue spots) were found inside the cytoplasm of epithelioid and Langhans giant cells (Lamina propria, ileum) [11].

Table 2: Analyzed samples and obtained results using four different diagnostic methods

Case	Organ	MAP culture	Histopathology	IHC	ISPCR
1	Ileum	Positive	Positive	+++	++
2	Ileum	Positive	Positive	+++	++
3	Ileum	Positive	Positive	+++	++
4	Ileocaecal LN	Positive	Positive	+++	++
5	Ileocaecal LN	Positive	Positive	+++	++
6	Ileum	Positive	Positive	+	++
7	Ileum	Positive	Positive	+++	++
8	Ileum	Positive	Positive	+++	++
9	Ileum	Negative	Negative	-	-

References: (IHC): immunohistochemistry, (dISP): direct in situ PCR. (+++): intense, (++) : moderate, (+): weak, (-): negative.

The PCR is performed by incubation of the sections with 50mL 1X reaction buffer (Gibco, BRL), 1.5U Taq polymerase, 2mmol/L MgCl<sub>2</sub>, 40mmol/L dNTP, 0.2mmol/L dUTP labelled with digoxigenin (Boehringer Mannheim, Lewes, UK) and 60 pg each of IS900 *M. avium* sub sp. *paratuberculosis* insertion sequence primers. The primers used are p89 (Sequence 5' CGTCGGGTATGGCTTTCATGTGGTTGCTGTG-3') and p92 (Sequence 5' CGTCGTTGGCCACCCG CTGCGAGAGCAAT-3'), previously tested by ISH[9;10]. The slides are sealed with the Assembly tool (Perkin Elmer, Cambridge, UK) and placed in a Touch Down thermocycler (Hybaid, Ashford, UK). PCR is undertaken with the following thermocycler conditions: 5min at 95°C, 35 cycles of 94°C (1min), 64.5°C (1min) and 72°C (1min) ending at 72°C for 2min. PCR products are detected with alkaline phosphatase- conjugated sheep antibodies against antidigoxigenin (Boehringer Mannheim) diluted 1/500. The chromogen is 5-bromo-4-chloro-3-indolyl phosphate toluidine salt and tetrazolium nitroblue (Boehringer Mannheim) diluted 1/50. Sections are counterstained with nuclear fast red [11].

**Application of Direct in Situ PCR:** Detection of Map in tissue samples supports pathological diagnosis and allows to determinate where the agent persists in the

experimental disease [9, 14]. The dISP was able to detect Map in all formalin-fixed, paraffin-embedded tissue samples from naturally infected cattle. The blue positive signal was clearly identified in areas with pathological changes, inside the macrophages or Langhans giant cells [11].

Detection of Map in tissue samples supports pathological diagnosis and allows to determinate where the agent persists in the experimental disease [9, 14]. The obtained results indicated that dISP was able to detect Map in all formalin-fixed, paraffin-embedded tissue samples from naturally infected cattle. The blue positive signal was clearly identified in areas with pathological changes, inside the macrophages or Langhans giant cells. Negative control and positive samples incubated without Taq enzyme resulted negatively, which confirmed that obtained signal in the positive samples was due to the presence of Map [11].

**Advantage and Disadvantage of Direct in Situ PCR:** It was described that spheroplasts may be related to the development of PTB or CD and these forms of Map can be detected by ISH [12, 14]. In a similar way, dISP would be useful for their identification since it is based in the detection of mycobacterial DNA and this fact may improve its sensitivity when compared to IHC [8].

Although the sensitivity of dISP was described as very high [58], Delgado *et al.* [11] results indicate that its sensitivity is lower than IHC since the dISP detected signal was moderate and immunostaining was intense in most of cases. However, this difference on staining results may be related with damage on DNA probably occurred during the fixation process, because variables such as time of fixation or nature of fixative solution were not considered when sampling was done and they can alter the DNA integrity [59]. Although IHC may also be affected by fixation, antigen recovery may recuperate immunogenicity considering that the analyzed samples were not collected for dISP and fixation was not controlled, further studies will be necessary to evaluate sensitivity of dISP [60].

### CONCLUSION AND RECOMMENDATIONS

Map detection by means of bacterial culture in solid medium is still the reference diagnostic method because it also allows categorizing the animals' slow, 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. Definitive diagnosis is made post-mortem by the signs found in the gastrointestinal tract, for which older animals are preferably selected.

Johnin skin test should be noted that positive reactions in deer may take the form of diffuse plaques rather than discrete circumscribed swellings, thus making reading of the test more difficult. The presence of any swelling should be regarded as positive in this species. However, sensitization to the *M. avium* complex is widespread in animals and neither avian tuberculin nor johnin are highly specific. Furthermore, the interpretation of the skin test results is complicated by the lack of agreement with respect to interpretation criteria.

New diagnosis technologies are in continuing development. However, histopathology and in situ detection techniques are useful tools, especially when molecular biology techniques or Map culture cannot be performed. IHC and ISH were suitable for the detection of Map in formalin fixed and paraffin embedded tissue samples.

Histopathology is used as a diagnostic method, but it is also a very important tool for researching PTB. Detection of Map in tissue samples increases the pathologic diagnosis and may be necessary when

experimental infections are performed. Several techniques such as Ziehl Nielsen staining (ZN), immunohistochemistry (IHC) and in situ hybridization (ISH) were tested for detection of the agent, but their performances are different. ZN and IHC are easy to perform and have high, but false negative can arise when infection was recent or bacilli were scanty. Besides, their specificity may be considered low since ZN cannot differentiate among acid fast microorganisms and antigens shared by different mycobacteria may affect IHC performance. When both are compared, ZN is cheaper, but IHC may detect antigens of Map even when the bacillus was digested in the cytoplasm of macrophages. On the other hand, ISH is specific but more expensive, hard to perform and its interpretation may be difficult because of the lower signal obtained. However, detection of Map by ISH is considered useful because it detects spheroplasts (Forms of Map with deficiencies in the cell wall) which may be involved with the disease and may be not detected by ZN or IHC. Besides, detection of DNA of Map may be useful when IHC and ZN are negative because bacilli and their antigens are damaged.

A direct in situ PCR method against IS900 DNA sequence was successfully used for Map detection in formalin-fixed, paraffin embedded tissue samples, which were obtained from naturally infected adult cows. Although the signal was lower than IHC, further studies should be necessary to determinate sensitivity and specificity of the technique. As it was previously described for ISH, detection of Map DNA by dISP should be useful for the detection of spheroplasts. However, the tissue's structure was affected and its development was more difficult than IHC. At the light of these facts, this method should be performed after IHC failed to detect Map or to detect spheroplasts in tissue samples with compatible changes. Tissue morphology was affected when dISP was performed. This fact may be related to the enzymatic digestion and repeated exposition of the slides to high temperature in each PCR cycle since this problem was not detected with IHC. Because of this, interpretation of the latter was easier, while tissues damaged by dISP required repeating the performed test.

The signal obtained with dISP was higher than the weak signal reported for ISH method. The cause of this difference may be related to the amplification of DNA obtained by dISP. Besides, the size of the probe, which has to penetrate the cell to hybridize the target DNA in ISH, did not affect the efficiency of dISP since primers and dNTPs are very small and constantly available.

Formalin fixation can alter the DNA integrity which in turn could affect ISH performance. This fixative solution can also modify the structure of antigens in tissue samples. Although formalin fixation can reduce the efficiency of both techniques, IHC would give better results since antigen retrieval process can overcome some detrimental effects of fixation. This difference could be an important advantage of IHC.

ISH and IHC were able to detect Map in formalin fixed paraffin embedded tissue samples although IHC resulted in higher intensity staining and was easy to carry out. Both of them features represent a very important advantage for this technique, which would make it a better in situ detection method for the initial study of Paratuberculosis.

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. Based on the above facts, the following recommendations are suggested:

- ISH and direct in situ PCR should be considered as the methods choice for detection of spheroplasts (forms of MAP with deficiencies in the cell wall).
- IHC Should be considered as the method choice for detection of Map in formalin fixed and paraffin embedded tissue samples.
- Further research works should be carried out to evaluate sensitivity, specificity and possible alternate protocols for ISH, IHC and ISP techniques.
- Two or more diagnostic tests should be conducted to increase the specificity and sensitivity of the tests for detection of infected animals in the silent or subclinical periods.

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