The Diagnosis of Paratuberculosis in Livestock

INTRODUCTION

Although a number of different diagnostic methods exist to screen for, or confirm the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) in ruminants, it is of crucial importance to be aware of this organism's specific pathobiological principles which are broadly responsible for the challenges facing diagnosticians in the control of paratuberculosis. Unlike the majority of livestock diseases, paratuberculosis elicits an extremely slow onset of pathological changes to tissues as well as immune reactions in the infected animal. In practical terms this period of delayed reaction does not permit the diagnostician insight into the animal's infection status and is often incorrectly interpreted as poor sensitivity of the diagnostic test(s) applied. Other considerations, for not only farmers and veterinarians, but also responsible veterinary authorities, are the availability of specialised laboratory facilities, costs of tests and market incentives.

DIAGNOSTIC APPROACHES

The range of diagnostic approaches can be broadly classified into two categories, based on either the direct detection of the causative bacterium or the host's immune response to infection with MAP:

**DIAGNOSIS OF PARATUBERCULOSIS**

- Direct detection of causal bacterium
- Indirect detection of causal bacterium

1. Microscopy (ZN staining of smears)
2. Bacterial isolation in culture medium
3. DNA based (molecular) techniques
   a. PCR amplification
   b. RFLP typing
4. Post mortem with ZN, IHC or PCR confirmation.
5. Cell-mediated (immune response)
6. Serology (humoral immune response)
   a. CFT
   b. AGID
   c. ELISA
d. Other
1. Direct microscopic examination of faecal smears

Direct microscopic examination of faecal smears stained by the Ziehl-Neelsen method is an inexpensive and rapid method to identify MAP in live infected animals or affected tissues from necropsied animals but only if they are in the excretory phase of the disease and excrete large numbers of bacilli, at a minimum number of 10 colony-forming units per gram of faeces. The presence of other large, saprophyte, acid-fast bacilli may complicate a diagnosis.

2. Bacterial culture

Bacterial culture is considered the traditional diagnostic tool and the 'gold' standard against which all other tests are compared. Semi-automated or automated culture systems using liquid selective media have nowadays replaced the traditional isolation on solid Harold’s egg-yolk medium (HEYM) and allow detection of MAP on average within 6 – 8 weeks. Culture is routinely combined with identification of MAP by PCR amplification and excludes other mycobacteria. For MAP isolation from live animals a faecal sample of at least 10g of faeces kept at 4°C should reach the laboratory within four days of collection. For reasons unknown the bacterium is more difficult to culture from faeces of sheep than from cattle, even when bacteria can be demonstrated microscopically in acid-fast stained smears.

Although a costly procedure, culture offers the additional advantage of genetic typing of the bacterial strain involved in a particular outbreak, thus allowing trace back of infections to a specific herd or flock of origin (see point 3.b.) as well as scientific epidemiological studies which are imperative to the development of effective control measures in future. The culture method also permits up-scaling of the test by pooling faecal samples from e.g. five animals into one test (pooled faecal culture), rendering the test more economical (refer to Eamens in recent published findings below).

3. DNA based techniques

Molecular DNA screening is of great value in confirmation of a diagnosis, and also widely used to measure genetic relatedness among isolates of the same species. IS900, a repetitive DNA insertion element of 1,5 kb present in 15 – 20 copies of the MAP genome, is targeted in paratuberculosis, whilst IS902 is targeted for the M. avium isolates. A comparison of 16 rRNA sequences has shown that the 16S rRNA of MAP is 99,9 % homologous to M. avium and 98,1% homologous to Mycobacterium bovis. Furthermore, the use of DNA fingerprinting techniques based on IS900 as a target sequence has been particularly successful in characterizing MAP strains. Molecular techniques allow for the monitoring of both horizontal and vertical spread of infection, tracing the origin of imported infected animals and of the transmission of infection among and from wild ruminants.

a. PCR amplification

Polymerase chain reaction (PCR) constitutes the molecular process of amplifying a specific DNA sequence of the target organism, in this case MAP, to measurable quantities. This method, which has been applied in the detection of a rapidly increasing number of human and veterinary pathogens over the past decade is best used in combination with bacterial culture of MAP (see point 2.). Alternatively, it has been shown to detect MAP directly from faecal samples of animals with advanced disease. PCR has also been successfully used to amplify MAP from formalin fixed, paraffin-embedded tissue sections of infected animals.

In a recent laboratory study conducted at the ARC-Onderstepoort Veterinary Institute tissue sections from infected and healthy control sheep were comparatively examined by histopathology and PCR and showed that PCR yielded a higher sensitivity than histopathology (Michel, unpublished data). Another advantage of this PCR test applied to tissue sections is the short time it takes for completion (approximately 3 days). Its sensitivity in comparison to culture, however, still needs to be determined (refer to Schönhenričer et al below).

b. RFLP typing

The restriction fragment length polymorphism (RFLP) technique is a molecular technique that makes use of genetic markers to determine the degree of homology/variability between strains of pathogens. With the use of this technique two major groups of MAP
have been identified. One group consists of those strains exclusively recovered from sheep and goats, and the second group include strains recovered from cattle, and some strains from sheep and goats. A further application of this technique lies in the retrospective tracing of the origin of strains following the introduction of MAP into a herd/flock previously free of the infection.

4. Post mortem examination

The post mortem diagnosis of Johne’s disease is based on:
- The detection of specific macroscopic and microscopic lesions.
- The demonstration of intralesional MAP in impression smears or tissue sections.

a. Histopathological examination

Criteria accepted for a positive histological diagnosis include the presence of epithelioid and/or Langerhans’-type giant cells, and the demonstration of intracellular acid-fast bacilli in these cells. Histopathology alone would not allow for:
- Identification of the bacilli beyond genus level.
- In the very early stages of Johne’s disease only a few acid-fast bacilli may be present resulting in false negatives.

The diagnostic immunohistochemical demonstration of MAP antigen in macrophages and giant cells is of great value and it enables antigens to be associated with typical histological lesions. If available it is an inexpensive and relatively simple procedure to follow but may have some limitations (refer to SA Martinson et al below).

5. Cell-mediated immune response

Tests classified under this category are directed at the cell-mediated immunity and include the intradermal tuberculin skin test and the gamma interferon test. These are based on the premise that infected animals first develop a cell-mediated immune response (T-cell based) against mycobacterial disease which can be measured upon stimulation of that immune system.

- The intradermal tuberculin skin test has been used for many years, but is of little value due to cross-reactivity with other pathogenic and saprophytic Mycobacterium spp on the one hand, and low sensitivity on the other.
- The gamma interferon test measures gamma interferon, a cytokine produced by specifically sensitised lymphocytes. The same test system is used as bovine tuberculosis in cattle and buffalo, whereby the interpretation is geared towards the reaction against avian tuberculosis. It has the potential to identify sub clinical cases in the early stages of infection with a sensitivity of approximately 70% and a specificity of approximately 97%. A comparative field study conducted in South Africa has shown diagnostic value of this test especially in combination with ELISA assays.

6. Humoral immune response

Serological investigation (detection of serum antibodies) is valuable and most commonly used for screening flocks/herds for MAP, but one needs to keep in mind that there are shortcomings in both sensitivity and specificity.

- The results of serological tests are mostly of value when performed on a herd or flock basis and as an indicator of exposure to MAP.
- False-positive reactions may occasionally be seen due to cross-reactions with other microorganisms.
- False-negative responses may be seen due to an insufficient antibody level as seen in early infection.

a. Complement Fixation Test (CFT)

The CFT has remained the official test in many countries to certify animals (on a herd/flock basis) free of infection for export/import purposes. It is not very species-specific and has a limited sensitivity and specificity and is cross-reactive with the genera Corynebacterium sp, Nocardia sp and Rhodococcus sp.
b. Agar Gel Immunodiffusion Test (AGID test)

The AGID has a sensitivity of between 17 and 40%. In South Africa a sensitivity of 18% was found in a study in sheep (Michel & Bastianello). The low sensitivity renders this test overall unsuitable for detecting infected animals in pre-clinical stages of paratuberculosis. Since it is also very labour intensive and easily biased by subjective interpretation this test has been replaced by ELISA in most laboratories over the past years.

c. Enzyme-linked immunosorbent assay (ELISA)

The ELISA forms an integral part of modern paratuberculosis control programmes in the screening of herds/flocks as it offers acceptable diagnostic performance in combination with the option for high-throughput testing through automation. A number of different manufacturers of paratuberculosis ELISA systems exist. The different systems can differ significantly in their diagnostic performance (sensitivity versus specificity) and it is of paramount importance to select the test system best suited (or a combination of systems) for the intended purpose (refer to Van Weering et al, G. van Schaik et al, DL Clark et al, Nielsen SS et al and Pinedo PJ et al below). For best results it is recommended to conduct testing of a representative sub-sample of the population at regular intervals. In the textbook, Infectious Diseases of Livestock edited by Coetzee and Tustin, the types of diagnostic tests to be used at different clinical stages of a disease, and types of immune response present, have been clearly illustrated. Below is an adaptation of the diagrammatic presentation as published in this textbook.

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**RECENT PUBLISHED FINDINGS ON TEST STRATEGIES**

Many recent articles have been published on the effectiveness and application of these different tests in different combinations. A few examples of such studies, and their findings, are listed below and the reader is encouraged to refer back to statements in the above text where appropriate:

In a study by DL Clark et al faecal culture was evaluated against serum ELISA and direct faecal PCR. Serum and faecal samples of 250 dairy cattle of unknown infection status were investigated. Faecal culture, PCR and ELISA revealed 67/250 (26.8%), 74/250 (29.6%) and 25/250 (10%) to be shedding MAP respectively. Culture and PCR therefore seem to have been able to detect more positive animals compared to ELISA. Using culture as gold standard, the PCR was 70.2% sensitive and 85.3% specific, and ELISA 31.3% sensitive and 85.3% specific. When culture reported less than 10 colony-forming units, the sensitivity and specificity of PCR and ELISA were 57.1% and 85.3%, and 4.8% and 97.8% respectively.

When culture revealed the presence of 1 to 40 colony-forming units the sensitivity of PCR and ELISA were 75% and 50% respectively. When culture revealed the presence of 40 or more colony forming units, the sensitivity of PCR and ELISA were 100% and 86.2% respectively. Due to a lack of negative cultures the specificity could not be calculated. In a study by Eamens et al the optimum pooling rate for radiometric faecal cultures in low shedding cattle was investigated. They found that a pooling rate of 5 samples per pool was required to reliably detect infected low shedding cattle. MAP could be detected in more than 50% of stored faecal samples and it seems that a minimum incubation period of 10 weeks is required to be capable of detecting cattle shedding equal or less than 5x103 organisms per gram of faeces. In a study by SA Martinson et al paired samples of formalin-fixed, paraffin-embedded ileums and lymph nodes from 204 culled dairy cows were investigated for evidence of infection by MAP. 151/204 samples were from animals that were tissue culture positive for MAP, and 53/204 samples were from animals that were culture negative on tissue
and faecal samples. In the culture-positive animals MAP was isolated from 78 samples of ileum and from 107 samples of lymph node. Ziehl-Nieelsen (ZN) acid-fast and immunoperoxidase stained slides were also examined and acid-fast staining organisms were identified in 7 of 78 (8.97 %) and 6 of 106 (5.61 %) culture-positive ileum and lymph node samples, respectively. In comparison immunohistochemical (IHC) staining, of the same culture-positive tissues, confirmed infection in the ileum in 9 of 78 (11.54 %) and in the lymph node in 5 of 106 (4.67 %) of samples. All the tissues from the culture-negative group of animals tested negative by means of ZN and IHC staining. The authors concluded that the sensitivity of these 2 tests in detecting MAP in culled dairy cows was not significantly different with good to almost perfect agreement. Both these tests seem to be much less sensitive than bacterial culture, as it only detected less that 6 % of tissue positives, compared with culture positive tissues.

Nielsen SS et al attempted to determine the time from first detecting MAP-antibodies in milk by ELISA to start of MAP shedding in animals exhibiting different shedding patterns. This study was carried out over 3 years and included a total of 24 076 milk and 10 074 faecal samples, which were obtained from 1906 cows. These samples were tested using ELISA and faecal culture respectively. These animals were classified into 5 shedding groups based on repeated faecal culture as non-shedders (1512/1906 cows or 79.3 %), transient shedders (36/1906 or 1.9 %), intermittent shedders (1.37/1906 or 7.2 %), low shedders (143/1906 or 7.5 %), and high shedders (78/1906 or 4.1 %). It was found that 5 % of the transient shedders, 30 % of the intermittent shedders, 60 % of the low shedders and 70 % of the high shedders were ELISA positive at the date of first positive faecal culture, and as many as 28 % high shedders and 14 % low shedders were positive ≥1 year prior to the first time of detecting shedding. Faecal culture confirmed shedding within the first year after a positive ELISA result in 10 % of 328 cows with fluctuating ELISA. In comparison 35 % of 445 cows shed with the last two or more ELISA's positive. MAP antibodies therefore seemed to be generally detected prior to the onset of bacterial shedding, with differences between the various patterns of shedding, and it was concluded that a positive ELISA was useful for predicting which animals would subsequently become infectious.

Pinedo PJ et al studied the association between the results of a serum ELISA, faecal culture and nested PCR tests on milk, blood and faeces for MAP detection in dairy cows. Faeces, blood and milk samples were obtained from 328 lactating dairy cows kept in four known infected herds. They found that a total of 61 animals (18.6 %) tested positive when all the tests were interpreted in parallel. They found the agreement between results in different pairs of tests to be poor, slight and fair in two, five and three of the 10 possible combinations, respectively. Faecal culture and faecal PCR had the highest kappa coefficient (0.39), representing fair agreement beyond chance. The lowest agreement was seen between ELISA and blood PCR (0.036; poor agreement). Statistically significant associations (P < 0.05) between the following test pairs were seen with the Fisher's exact test: ELISA and faecal culture; ELISA and faecal PCR; milk and faecal PCR; blood PCR and faecal PCR; and faecal culture and faecal PCR. It was found that ELISA assays showed the highest complementary sensitivity values for all the possible two-test combinations, followed by faecal PCR, and the combined use of ELISA and faecal PCR seems to have potential for increasing the overall sensitivity for the diagnosis of paratuberculosis.

Schönenbrücher et al developed and validated a Taqman real-time PCR, which amplifies the F57 and the ISMv2 sequences of MAP from bovine faecal samples. Comparative analysis between this technique and culture of 108 naturally contaminated samples of unknown MAP status resulted in a relative accuracy of 98.9% and a sensitivity of 94.4% for faecal samples containing <10 CFU/g faeces. Sergeant et al evaluated five different herd-testing strategies in 19 Danish dairy herds which included a milk-ELISA on all lactating cows; a milk-ELISA on lactating cows of four to less than four years old; a milk-ELISA on lactating cows older than 4 years; a faecal culture on all lactating cows; and lastly a milk-ELISA plus faecal culture in series on all lactating cows.
They found whole herd milk ELISA as the preferred and most cost effective of the different strategies.

In a study by G. van Schaik et al, the authors attempted to investigate the diagnostic validity of pooling faecal samples and compared it to faecal culture and ELISA on individual samples to determine the cow or herd status for MAP. It was found that the sensitivity of the pools relative to individual faecal culture was 46% and 48% for pools of 5 and 10 cows, respectively. The sensitivity of the pools was lower when low shedder animals were tested, at 26% and 24% for pools of 5 and 10 samples respectively, compared to pools with moderate or heavy shedders (>75% sensitivity). It is recommended that a pool size of 10 is a better (more cost effective) option to determine, or monitor the herd status. They concluded that it seems that whole herd ELISA is the least expensive method of determining the status in individual cases, however, it has a lower sensitivity and specificity compared to individual culture. In a study by Van Weering et al was evaluated the diagnostic performance of a commercially available ELISA in individual and bulk milk samples.

Results were expressed as percentage S/P, calculated by 100 x (OD value of the sample - the OD value of the negative control) / (OD value of the positive control - the OD value of the negative control). Cut-offs as recommended by the manufacturer are for serum samples <60% S/P negative, 60-70% S/P ambiguous, and >70% S/P positive, and for milk samples <30% S/P negative, 30-40% S/P ambiguous, and >40% S/P positive. It was found that in individual milk samples, it had a specificity of 99.8% at a cut-off of 20% sample to positive (S/P). The relative sensitivity for individual milk samples, compared to positive serum samples was 87% for a cut-off of 20% S/P, and 80% for a cut-off of 30% S/P. The sensitivity to detect high shedders was > 90% for individual milk and serum samples. The agreement were very good (kappa = 0.91) for all paired samples. The specificity for testing bulk milk samples was 100% at a cut-off of 12.5% S/P but at the cut-off (30% S/P) recommended by the manufacturer performance of the bulk milk ELISA related to herd status (> or = 2 seropositive cows) was rather poor, corresponding with a sensitivity of 24% and a specificity of 99% relative to the serology. At a revised cut-off for bulk milk of 12.5% S/P and a within-herd seroprevalence of > or = 3%, the sensitivity and specificity, relative to the serology were 85% and 96%, respectively.

CONCLUSION

The few studies referred to above merely illustrates the many different options available, and differences in results that may be obtained, using different test strategies. In general for confirmation of a diagnosis of Johne's disease in a herd, regardless of the detection methods chosen it should, where possible, be supported by a direct detection method. Due to their low costs, serological tests seem very suitable as herd tests. A sensitive, practical and economical strategy for the diagnosis of paratuberculosis may be a combination of an ELISA and strategically pooled faecal cultures/PCR.

REFERENCES