

# Molecular Diagnostic to Identify Mycobacterial Species in Veterinary Science

Carlos Julian De Luna, Olivier Andre Ettore Sparagano\*

## Abstract

The genus *Mycobacterium* is of utmost importance in veterinary medicine. Members of the genus cause serious diseases to livestock and they also could be transmitted to humans. Bovine tuberculosis, principally caused by *M. bovis*, is a widespread disease and causes serious economic losses to farmers worldwide. Paratuberculosis or Johne's disease caused by the *M. avium* complex, is a slow-developing disease present in several species, particularly cattle, and it has been associated with Chron's disease of humans. It causes diarrhoea, and weight loss that could be fatal. The common diagnostic methods used rely upon culturing and microbiological identification of the *Mycobacterium* involved. However, the pathologically important species of *Mycobacterium* grow very slowly and could take up to eight to ten weeks to successfully grow on plates. Tuberculin and other immunological response tests are also widespread. However, these techniques also take long time to develop and often they are not conclusive. Recently molecular techniques based on the detection of *Mycobacterium* nucleic acids and proteins have proven a reliable and more important fast method for the diagnosis of *Mycobacterium*. Some tests can diagnose the presence of the pathogens in a couple of days. We reviewed the molecular methods available from the scientific literature that have reported to be useful in veterinary science.

---

**Keywords :** *Mycobacterium*, bovine tuberculosis, paratuberculosis, molecular diagnostic.

---

School of Agriculture, Food and Rural Development, Agriculture Building, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK.  
Olivier.Sparagano@ncl.ac.uk

\*Corresponding author

## Introduction

The genus *Mycobacterium* causes several diseases of veterinary importance. It has been the subject of much scientific research. For instance, the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>) holds, at the time of submitting this paper, the number of deposited sequences for *Mycobacterium tuberculosis* is 8831, whereas for *M. bovis* is 845 and for *M. avium* is 3075 sequences. The tuberculosis complex (MTBC), formed by *Mycobacterium tuberculosis* and *M. bovis*, is an infectious disease that is distributed worldwide. Although it was once controlled, especially in the developing world, in recent years an increase on the incidence of cases has been reported (DEFRA, 2006<sup>a</sup>). Bovine tuberculosis causes serious economic losses to farmers worldwide; the World Health Organization has estimated the economic losses to be around \$3 billion worldwide. Bovine tuberculosis is particularly important since it could present a high risk of transmission to humans. The principal way of infection to humans is via the consumption of non-pasteurised milk or dairy products from infected cows. In many countries bovine tuberculosis is a major infectious disease among cattle, other domesticated animals, and among certain wildlife populations. In recent years an increase on cases of tuberculosis has been reported and is found to be widespread in cattle (DEFRA, 2006<sup>a</sup>).

*Mycobacterium avium* subspecies *paratuberculosis* (MAP) causes paratuberculosis or Johne's disease, an intestinal granulomatous infection most often found among domestic and wild ruminants and has a global distribution. Although it is present worldwide, it is particularly more important in temperate regions of the developed world. Methods of control have proven difficult because of the long time spent between infection and the presentation of clinical signs (DEFRA, 2006<sup>b</sup>). Like most mycobacteria MAP is difficult to treat, it has developed resistance to antibiotics and, normally treatments to cure paratuberculosis last for extended periods of time.

Paratuberculosis is also responsible of causing serious economic losses to the agricultural industry, in the US economic losses to the cattle industry are around \$1.5 billion annually (Harris and Barletta, 2001). Vaccines for both diseases are currently under research, using several antigens as vaccine candidates.

Traditionally, mycobacteria are identified by phenotypic traits, such as morphological features, growth rates, preferred growth temperature, pigmentation and biochemical profiles (Yam et al., 2006). These tests are laborious, time consuming and the diagnosis takes time. During the last decades, novel techniques have been implemented in order to decrease the time from weeks to days to reach the conclusive diagnosis. Here we review the molecular techniques available on the scientific literature that have proven useful for the identification of mycobacterial pathogens.

## Molecular diagnosis within the *Mycobacterium* genus. Restriction Fragment Length Polymorphism

Gurtler et al. (2006) developed a technique that relies on the partial amplification of the Intergenic Spacer Region (ISR) of the 16S-23S rDNA gene and the consequent digestion of the amplicons by Restriction Fragment Length Polymorphism (RFLP) to diagnose clinically important *Mycobacterium*. An amplicon of around 220 bp, which was typically found on slow growing mycobacteria, was first amplified and later digested with the restriction enzymes Sau96I and HaeIII in order to distinguish among the following mycobacteria: *M. bovis*, *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. goodii*, and the *Mycobacterium avium* complex.

## Hypervariable regions of the 16S rRNA gene.

The use of rRNA hypervariable regions has been used for the identification of species-specific bacteria. Nine hypervariable regions (V1-V9) have been found in the bacterial 16S rRNA gene. The sequence analysis of the hypervariable region 2 (V2) has proven useful

for the identification of the genus *Mycobacterium* to the species level (Chakravorty et al., 2007). V2 showed an average length of 100 bp. A separate analysis of three 15–35 bp regions within V2 revealed that the 13 nucleotides spanning 182–194 contained the maximum single nucleotide polymorphisms (SNP) variations between *Mycobacterium* species (Chakravorty et al., 2007).

#### **Molecular methods for the diagnosis of the MTBC.**

Traditional diagnosis of bovine tuberculosis is confirmed through post-mortem examination and bacteriological culture of *M. bovis* organisms. The polymerase chain reaction (PCR) has been widely used for the detection of MTBC in clinical samples (mainly sputum) in human and animals. A number of commercially available kits and various “in-house” methods have been used for the detection of the MTBC in fresh and fixed tissues (Miller et al., 1997). Various primers have been used, including primers that have amplified sequences from 16S–23S rRNA, the insertion sequences IS6110 and IS1081, and genes coding for MTBC -specific proteins, such as MPB70 and the 38 kDa antigen b (Wiker et al., 1998).

However, commercial kits using fresh, frozen or boric acid-preserved tissues, have shown variable and less than satisfactory results in interlaboratory comparisons (Noredhoek et al., 1996). PCR is not only used for direct detection in material, or strain characterisation or differentiation within the MTBC complex, but it is also widely used as a method of initial identification. Usually some biochemical tests are done to confirm the finding. However, PCR is now being used on a routine basis to detect the MTBC and distinguish it from *M. avium* in formalin-fixed, paraffin-embedded tissues (Miller et al., 1997; Miller et al., 2002). Optimal results are obtained when both PCR and isolation methods are used.

DNA analysis techniques may prove to be faster and more reliable than biochemical methods for the differentiation of *M. bovis* from other members of the MTBC. A mutation at nucleotide position 285 in the

*oxyR* gene has been found to be specific for *M. bovis* in all MTBC isolates examined to date (Espinosa de los Monteros et al., 1998).

Genetic fingerprinting allows the distinction between different strains of *M. bovis*. The most widely used method is spoligotyping (from “spacer oligotyping”), which detects the differentiation of strains inside each species belonging to the MTBC, including *M. bovis*, and can also distinguish *M. bovis* from *M. tuberculosis* (Heifets and Jenkins, 1998).

Other techniques that have been used to differentiate more accurately the strains that have the same spoligotype include restriction fragment length polymorphism (RFLP) using IS6110, the direct repeat (DR) region and DNA probes (Skuce et al., 1996), RFLP using a combination of the DR and probes (O’Brian *et al.*, 2000) and characterisation of the variable number of tandem DNA repeat (VNTR) profile (Frothingham and Meeker-O’Connell, 1998).

Cobos-Marin et al. (2003) utilised a multiplex PCR to identify *M. bovis* in clinical isolates from other members of the *M. tuberculosis* complex. The method relies on a single PCR followed by a multiplex PCR. The single PCR reaction was used to make a genus identification of the *Mycobacterium spp.* A set of primers (RAC1/RAC8) coding for the *mur A* gene, the promoter region of the rRNA operon, and the 5’end of the 16S rRNA gene were used for the genus identification. This set was then multiplexed using another set of primers (Y277-32F/Y277-32R) that also amplified another gene fragment of the promoter region of the rRNA operon and the 5’end of 16S rRNA gene. The products, when electrophoresed and visualised on an agarose gel, generated a single band for *M. bovis* and two bands if they belonged to another member of the *Mycobacterium spp.* (Cobos-Marin et al., 2003).

#### **Molecular methods for the diagnosis of *M. avium*.**

**DNA probes:** DNA probes have been developed to detect MAP in diagnostic samples and of rapidly

identifying bacterial isolates (Ellingson et al., 1998). They have been used to distinguish between MAP and other mycobacteria, especially those of the *M. avium* complex, (MAC) which also includes *M. gordonae*. McFadden et al. (1987) identified a sequence named IS900, which is an insertion sequence specific for MAP. The use of IS900 as a DNA probe for specific identification of MAP in faecal samples from cattle by amplification of DNA has also proven useful in later studies (Vary et al., 1990; Marsh et al., 2000). Therefore, a commercial diagnostic test based on the detection of IS900 sequences following isolation of mycobacteria from faecal samples and enrichment of a DNA fraction from IS900 sequences by PCR has been developed. The test is available from a commercial laboratory (IDEXX, <http://www.idexx.com>). Reverse cross blot hybridisation and sequence analyses allowed the identification of other members of the genus *Mycobacterium* from those of the MTBC (Hughes et al., 2005). The presence of the genes for potential diagnostic antigens: MPB70, MPB64, ESAT-6 and CFP-10 in the isolated *Mycobacterium* species. Molecular analyses of cultured isolates from bovine lymph node specimens of 48 cattle identified a wide variety of mycobacterial species including MAP (Hughes et al., 2005).

### Real-time quantitative PCR

*Mycobacterium*-specific real time qPCR assay has been successfully used in combination with *Mycobacterium*-specific 16S rRNA sandwich hybridization in culture-independent analysis of reservoirs of viable and potentially infectious mycobacteria (Pakarinen et al., 2007). A molecular beacon-based real-time NASBA assay for detection and identification of MAP has been developed (Rodriguez-Lazaro et al., 2004). It targets and amplifies sequences from the *dnaA* gene which are specific for this bacterium. The assay was tested against 18 isolates of MAP, 17 other mycobacterial strains and 25 non-mycobacterial strains, and was fully selective in that it detected all the targets but none of the non-targets

(Rodriguez-Lazaro et al., 2004).

### Diagnostic using postgenomic techniques

A novel approach uses the detection of specific proteins present in MAP. Among those proteins, a secretome database has been created, and from the database 25 candidate the diagnosis of MAP could be performed, these antigens were then analysed by immunoproteomic analyses and found to have a high sensitivity and specificity in the detection of MAP (Leroy et al., 2007).

In summary, molecular techniques have been used for several years now, and they offer a practical and rapid method of detection of *Mycobacterium* spp. and in the long run, it may also be cheaper to run molecular techniques when compared with the associated costs of running serological, immunological and microbiological tests. Improvement in the reliability of PCR as a practical test for the detection of the MTBC in fresh clinical specimens will require the development of standardised and robust procedures.

### References

- Chakravorty, S., Helb, D., Burday, M., Connell, N. and Alland, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *J. Microbiol. Meth.* 69: 330-339.
- Cobos-Marin, L. Montes-Vargas, J., Rivera-Gutierrez, S., Licea-Navarro, A., Gonzalez-Y-Merchand, J.A. and Estrada-Garcia, I. 2003. A novel multiplex-PCR for the rapid identification of *Mycobacterium bovis* in clinical isolates of both veterinary and human origin. *Epidemiol. Infect.* 130: 485-490.
- DEFRA (Department of the Environment, Food and Rural Affairs) 2006<sup>a</sup>. "Provisional profile for bovine tuberculosis". [Online]. Available at <http://www.defra.gov.uk/animalh/diseases/vetsurveillance/profiles/bovinetb.pdf>

- DEFRA (Department of the Environment, Food and Rural Affairs) 2006b. “Provisional profile for Johne’s disease”. [Online]. Available at <http://www.defra.gov.uk/animalh/diseases/vetsurveillance/profiles/johnes.pdf>
- Ellingson, J.L.E., Bolin, C.A. and Stabel, J.R. 1998. Identification of a gene unique to *Mycobacterium avium* subspecies *paratuberculosis* and application to diagnosis of paratuberculosis. *Mol. Cell. Probes*, 12: 133-142.
- Espinosa de los Monteros, L.E., Galan, J.C., Gutierrez, M., Samper, S., Garcia Marin, J.F., Martin, C., Domínguez, L., de Rafael, L., Baquero, F., Gomez-Mampaso, E. and Blázquez, J. 1998. Allele-specific PCR method based on *pncA* and *oxyR* sequences for distinguishing *Mycobacterium bovis* from *M. tuberculosis*: intraspecific *M. bovis pncA* sequence polymorphism. *J. Clin. Microbiol.* 36: 239-242.
- Frothingham, R. and Meeker-O’Connell, W.A. 1998. Genetic diversity in the *Mycobacterium tuberculosis* complex based on variable numbers of tandem DNA repeats. *Microbiol.* 144: 1189-1196.
- Gurtler, V., Harford, C., Bywater, J. and Mayall B.C. 2006. Direct identification of slowly growing *Mycobacterium* species by analysis of the intergenic 16S-23S rDNA spacer region (ISR) using a GelCompar II database containing sequence based optimization for restriction fragment site polymorphisms (RFLPs) for 12 enzymes. *J. Microbiol. Meth.* 64: 185-199.
- Harris, N.B. and Barletta, R. G. 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. *Clin. Microbiol. Rev.* 14 (3): 489-512.
- Heifets, L.B. and Jenkins, P.A. 1998. Speciation of *Mycobacterium* in clinical laboratories. In: *Mycobacteria I. Basic Aspects*, Gangadharam, P.R. and Jenkins, P.A., (eds.) New York, USA: Chapman and Hall. 308-350.
- Hughes, M.S., Ball, N.W., McCarroll, J., Erskine, M., Taylor, M.J., Pollock, J.M., Skuce, R.A., and Neill, S.D. 2005. Molecular analyses of mycobacteria other than the *M. tuberculosis* complex isolated from Northern Ireland cattle. *Vet. Microbiol.* 108: 101-112.
- Leroy, B., Roupie, V., Noël-Georis, I., Rosseels, V., Walravens, K., Govaerts, M., Huygen, K. and Wattiez, R. 2007. Antigen discovery: a postgenomic approach to paratuberculosis diagnosis. *Proteomics*. 7: 1164-1176.
- Marsh, I., Whittington, R. and Millar, D. 2000. Quality control and optimized procedure of hybridization capture-PCR for the identification of *Mycobacterium avium* subsp. *paratuberculosis* in faeces. *Mol. Cell. Probes*. 14: 219-232.
- McFadden, J.J., Butcher, P.D., Thompson, J., Chiodini, R. and Hermon-Taylor, J. 1987. The use of DNA probes identifying restriction fragment length polymorphisms to examine the *Mycobacterium avium* complex. *Mol. Microbiol.* 1: 283-291.
- Miller, J., Jenny, A., Rhgyan, J., Saari, D. and Saurez, D. 1997. Detection of *Mycobacterium bovis* in formalin-fixed, paraffin-embedded tissues of cattle and elk by PCR amplification of an IS6110 sequence specific for *M. tuberculosis* complex organisms. *J. Vet. Diagn. Investig.* 9: 244-249.
- Miller, J., Jenny, A. and Payeur, J. 2002. Polymerase chain reaction detection of *Mycobacterium bovis* and *M. avium* organisms in formalin-fixed tissues from culture-negative organisms. *Vet. Microbiol.* 87(1): 15-23.
- Noredhoek G.T., van Embden J.D.A. and Kolk A.H.J. 1996. Reliability of nucleic acid amplification for detection of *Mycobacterium tuberculosis*: an international collaborative quality control study among 30 laboratories. *J. Clin. Microbiol.* 34: 2522-2525.

- O'Brian, R., Danilowicz, B.S., Bailey, L., Flynn, O., Costello, E., O'Grady, D. and Rodgers, M. 2000. Characterisation of the *Mycobacterium bovis* restriction fragment length polymorphism DNA probe pUCD and performance comparison with standard methods. J. Clin. Microbiol. 38: 3362-3369.
- Pakarinen, J., Nieminen, T., Tirkkonen, T., Tsitko, I., Ali-Vehmas, T., Neubauer, P. and Salkinoja-Salonen, M.S. 2007. Proliferation of mycobacteria in a piggery environment revealed by mycobacterium-specific real-time quantitative PCR and 16S rRNA sandwich hybridization. Vet. Microbiol. 120: 105-112.
- Rodriguez-Lazaro, D., Lloyd, J., Herrewegh, A., Ikononopoulos, J., D'Agostino, M., Pla, M. and Cook, N. 2004. A molecular beacon-based real-time NASBA assay for detection of *Mycobacterium avium* subsp. *paratuberculosis* in water and milk. FEMS Microbiol. Lett. 237: 119-126.
- Skuce, R.A., Brittain, D., Hughes, M.S. and Neill, S.D. 1996. Differentiation of *Mycobacterium bovis* isolates from animals by DNA typing. J. Clin. Microbiol. 38: 2469-2474.
- Vary, C.P.H., Anderson, P.R., Green, E., Hermon-Taylor, J. and McFadden, J.J. 1990. Use of highly specific DNA probes and the polymerase chain reaction to detect *Mycobacterium paratuberculosis* in Johne's disease. J. Clin. Microbiol. 28: 268-275.
- Wiker, H.G., Lyashchenko, K.P., Aksoy, A.M., Lightbody, K.A., Pollock, J.M., Komissarenko, S.V., Bobrovnik, S.O., Kolesnikova, I.N., Mykhalsky, L.O., Gennaro, M.L. and Harboe, M. 1998. Immunochemical characterization of the MPB70/80 and MPB83 proteins of *Mycobacterium bovis*. Infect. Immun. 66(4):1445-1452.
- Yam, W.C., Yuen, K.Y., Kam, S.Y., Yiu, L.S., Chan, K.S., Leung, C.C., Tam, C.M., Ho, P.O., Yew, W.W., Seto, W.H. and Ho, P.L. 2006. Diagnostic application of genotypic identification of mycobacteria. J. Med. Microbiol. 55: 529-536.