Validation and improvement of mycobacterial diagnostic methods - Pandolfi J.R.[1]*, Silva V.S.[1], Kramer B.[1], Araújo F.R.[2], Peixoto J.O.[1], Coldebella A.[1], Lopes L.S.[1], Ibelli A.M.G.[1], Tessmann A.L.[1], Brentano L.[1], Magarelli G.[3]

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Animal tuberculosis is a chronic zoonotic disease caused by *Mycobacterium bovis*. Isolation of the etiological agent is the gold standard in the diagnosis of animal tuberculosis. However, it has limitations due to fastidious bacillary growth and in sample preparation and decontamination process. Thus, diagnosis can takes months to be finished. Such diagnostic delay produces serious sanitary and production losses. Several new tests are being developed but they lack ideal sensitivity and specificity. Hence, it is important to improve current diagnostic tests. Thus the aim of this study is to standardize and improve classical and molecular methods of *in vitro M. bovis post-mortem* diagnosis. This study will revise classical methods (sample preparation, decontamination and mycobacterial isolation) and molecular methods (PCRs for amplification of specific DNA fragments of mycobacteria and techniques to identify the mycobacterial species by using restriction endonucleases in the PCR products). Employing the reference strains of mycobacteria (*M. bovis, M. avium hominisuis*) in serial dilutions from the initial suspension corresponding to 1 on Mac Farland standards, in triplicate, each medium will be evaluated on their ability to promote mycobacterial growth. For molecular diagnosis, the same dilutions described previously will be used for DNA extraction by thermolysis and direct use in PCR mix. Different commercial polymerases will be tested. The reactions will be performed in triplicate. After the standardization of techniques, improvement of these methods will be done, employing inoculated tissue samples (lung tissue from SPF pigs). This bench validation will be followed by further field studies.

Key-words: *Mycobacterium bovis*, PCR, identification, tuberculosis

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Validation and improvement of mycobacterial diagnostic methods

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Introduction

Animal tuberculosis is a chronic zoonotic disease caused by *Mycobacterium bovis*. Isolation of the etiological agent is the gold standard in the diagnosis of animal tuberculosis. However, it has limitations due to fastidious bacillary growth and in sample preparation and decontamination process. Thus, diagnosis can takes months to be finished. Such diagnostic delay produces serious sanitary and production losses. Several new tests are being developed but they lack ideal sensitivity and specificity. Hence, it is important to improve current diagnostic tests. Thus the aim of this study is to standardize and improve classical and molecular methods of *in vitro M. bovis post-mortem* diagnosis, to be done at Embrapa Swine and Poultry Biosecurity Level 3 Laboratory (NB3) (figure 1).

Figure 1. Embrapa Swine and Poultry Biosecurity Level 3 Laboratory (NB3). **A**. Lab. entrance; **B**. Mycobacteriology; **C**. Molecular Biology.

Materials & Methods

This study will revise classical methods (sample preparation, decontamination and mycobacterial isolation) and molecular methods (PCRs for amplification of specific DNA fragments of mycobacteria and techniques to identify the mycobacterial species by restriction endonucleases in the PCR products).

The study will be performed on two steps. The first one will employ cultured micobacteria and the second will be done using tissue samples from SPF swines, artificially infected.

Micobacteria Strains

All procedures will be performed employing standard strains *(M. bovis* AN5, *M. avium avium* D4, *M. bovis* BCG Moreau, *M. avium hominisuis).*

Classical Methods

Sample preparation

Samples from lung and lyphonodes will be used.

Mycobacterial isolation

Isolation will be done on liquid (Middlebrook 7H9 - enriched with commercial or homemade OADC and/or glicerol-) and solid media (LJ -enriched with glicerol or piruvate-, Stonebrink and Ogawa-Khudo) (Figure 2).

Employing the reference strains of mycobacteria (*M. bovis, M. avium hominisuis*) in serial dilutions from the initial suspension corresponding to 1 on Mac Farland standards, in triplicate, each medium will be evaluated on their ability to promote mycobacterial growth.

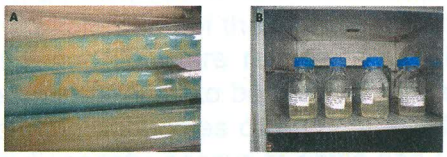

Figure 2. Mycobacterial cultures. **A**. Lowenstein-Jensen; **B**. Middlebrook 7H9.

Molecular Methods

For molecular diagnosis, the same dilutions described previously will be used for DNA extraction by thermolysis and direct use into PCR mix. Different commercial polymerases will be tested. The reactions will be performed in triplicate. After the standardization of techniques, improvement of these methods will be done, employing inoculated tissue samples (lung tissue from SPF pigs).

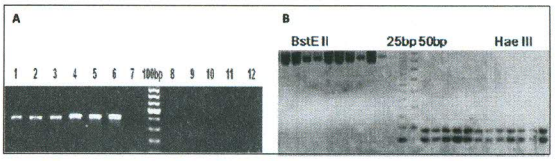

Figure 3. Examples of PCR for Mycobacterial identification. **A**. PCR specific for Mycobacterium genus – amplifies *hsp*65 gene; **B**. hsp65 PCR - Restriction enzyme Analysis (PCR-PRA) – a restriction fragment lenght polimorphism technique for mycobacterial species identification.

Future Directions

This bench validation will be followed by further field studies on future projects.