

Full Length Research Paper

## Detection of *Mycobacterium bovis* in bovine carcasses by multiplex-PCR

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The causative agent of bovine tuberculosis (BTB) is *Mycobacterium bovis*, a bacterium belonging to the *M. tuberculosis* complex (MTC). The definitive diagnosis is achieved through isolation and identification of *M. bovis* from clinical samples, using a combination of traditional culture and biochemical methods, which is considered the “gold standard”. This procedure is cumbersome and time-consuming. We evaluated a multiplex-PCR (m-PCR) assay for the direct detection of *M. bovis* DNA from tissue with BTB-suspected lesions. A dairy herd consisting of 270 adult cattle where 34 animals were positive to the tuberculin skin test has been reported. At 30 days after the tuberculin test, all 34 reactive animals were slaughtered and subjected to a necropsy procedure. A pool of tissue samples representative of each animal (lung and mediastinal, scapular and retropharyngeal lymph nodes) were collected and subjected to bacteriological culture and m-PCR. *Mycobacterium* spp. was isolated in 50% (17/34) of the collected samples. When using m-PCR directly from tissue fragments, it was possible to detect *M. bovis* in 67.6% (23/34) of the collected samples including 15 samples isolated by bacteriological culture. High performance liquid chromatography (HPLC) was used to differentiate the 17 isolated strains of *Mycobacterium* spp., from the *Mycobacterium tuberculosis* complex (MTC) or other *Mycobacterium* sp. not belonging to the MTC. The use of m-PCR assays directly from tissue samples may be a valid supplementary tool for the *post mortem* diagnosis of BTB, since this is a faster and more specific technique than bacterial culturing, reducing the diagnosis time for diagnosis of the disease from three months to two days.

**Key words:** Bovine tuberculosis, high performance liquid chromatography (HPLC), *Mycobacterium tuberculosis* complex, multiplex polymerase chain reaction (PCR), tissues with macroscopic lesions.

## INTRODUCTION

Bovine tuberculosis (BTB) is a major infectious disease among cattle in many countries. Although cattle are the main host and reservoir of this chronic infection, other mammals, including humans, are also susceptible to *Mycobacterium bovis* (Medeiros et al., 2010). Zoonotic TB can also be considered a socio-economic disease, as it causes direct economic losses in the agribusiness and hampers commercial exchange of animals and products (Zumárraga et al., 1999). Many countries around the world perform the control or eradication of BTB by their official control of infectious diseases, based on test-and-slaughter policy. Brazilian policies regarding the control and eradication of BTB include the National Plan for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT), established in 2001 and reviewed in 2004, which is based on the slaughtering of all reactive animals to the tuberculin test (Brazil, 2006). According to Pollock et al. (2005) new tools, such as additional diagnostic tests, are needed to make a quick diagnosis of the disease and develop vaccines in order to prevent bovine tuberculosis.

There is a lack of official data regarding the current prevalence of BTB in Brazil. Based on official reports, there was a national average prevalence of 1.3% of cows infected from 1989 to 1998 (Brazil, 2006). Since the implementation of the PNCEBT in Brazil, the prevalence of the disease was reported to range from 0.7 to 3.3% (Furlanetto et al., 2012). According to the epidemiology of the disease, there is a higher incidence of BTB in dairy herds when compared to beef herds, due to the difference between the breeding systems of these animals.

BTB is usually diagnosed “*in vivo*”, based on delayed hypersensitivity reactions (intradermal tuberculin tests), which may lack high sensitivity and specificity. However, a definitive diagnosis is still established by the isolation and identification of the etiological agent (*M. bovis*) from lymph nodes or lungs, obtained during necropsy or at slaughter, using a combination of traditional culture and biochemical methods, which is considered the “gold standard method”. These methods are laborious, unreliable and time-consuming; it may take more than 90 days to grow the microorganism, and an additional 2 weeks for biochemical identification (OIE, 2009). Several alternative approaches have been attempted for the rapid and specific diagnosis of BTB, but molecular methods, especially the polymerase chain reaction (PCR) assay, are the most promising (Carvalho et al., 2015).

BTB lesions in cattle are most often found in organs rich in reticuloendothelial tissue, particularly the lungs and associated lymph nodes (Corner et al., 1990). Other

studies conducted on naturally infected cattle experimentally infected with *M. bovis*, demonstrated that lesions are most commonly present in the lower respiratory tract, however the upper respiratory tract and its associated tissues also displays disease in many cases (Neill et al., 1994; Rodgers et al., 2007). Although tubercles are not pathognomonic of BTB, identifying *M. bovis* or its DNA confirms the disease.

PCR has been successfully applied by our group and other researchers in the detection of members from the *M. tuberculosis* complex (MTC), and DNA amplification of specific sequences is especially useful for this (Cardoso et al., 2009; 2015). However, the success of the PCR assay depends on the availability of intact and impurity-free DNA. Thus the presence of contaminants can interfere with the PCR technique, becoming an obstacle for its implementation (Cardoso et al., 2009). Vitale et al. (1998) showed that the QIAamp Blood and Tissue Kit (Qiagen®) was able to circumvent these problems, supplying DNA templates suitable to be amplified by PCR in most biological samples. We adopted this procedure to evaluate the efficiency of an m-PCR targeting for the *RvD1Rv2031c* and *IS6110* sequences, specific for *M. bovis* and MTC, respectively, to identify *M. bovis* DNA from tissues of slaughtered, skin-test positive, animals. The results were compared with those obtained from the skin test and conventional culture for *M. bovis*.

## MATERIALS AND METHODS

### Study design

This study was conducted on a dairy herd comprised of 270 adult crossbred Holstein and Gir cows, located in Macaé city, Rio de Janeiro State, in Southeastern of Brazil. Prior to the study, 34 adult cows had positive reactions to a single intradermal tuberculin test (SITT) and were kept in quarantine for 90 days, waiting for confirmatory tests to be conducted, in order to avoid bacillus transmission. After 90 days, a comparative intradermal tuberculin test (CITT) was performed in these same 34 cows (Group A - reagents), plus 16 randomly selected cows that were negative to the first SITT test (Group B - control), totaling 50 animals. After 30 days of the PPD injection, all CITT-reactive cattle (Group A) were slaughtered and subjected to a necropsy procedure (OIE, 2009). Mediastinal, scapular and retropharyngeal lymph nodes, as well as lung samples of lungs, independently of the macroscopic tuberculous lesions, were collected and analyzed by bacteriological culturing and PCR.

### Intradermal tuberculin test

Intradermal tuberculin tests (both SITT and CITT) for BTB diagnosis were performed on all 50 cows, in accordance with the regulations of the Ministry of Agriculture, Livestock and Supply (Brasil, 2006). For the SITT, 0.1 mL of bovine PPD (bovPPD-*M. bovis* strain AN5,

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1 mg protein/mL; Instituto Biológico, São Paulo - SP, Brazil) was inoculated in the cervical area of each cow. After 72 h, the inoculation site was measured with a caliper, and the cow was considered reactive if a swelling >4.0 mm occurred at the injection site. The CITT consisted of the same procedure, plus an inoculation of 0.1 mL avium PPD (*M. avium* strain D4, 0.5 mg protein/mL; Instituto Biológico) in the cervical area, approximately 20 cm from the boVPPD inoculation. Cattle were considered reactive if the difference between the thicknesses of both inoculation sites were >4.0 mm.

### Isolate culturing and identification

All CITT-reactive cows were killed 30 days after the PPD injection, and a thorough necropsy was conducted. Mediastinal, scapular and retropharyngeal lymph nodes, as well as lung samples, independent of macroscopic tuberculous lesions, were collected. A total of four tissue fragments were collected per animal. A pooled sample from each animal was packed in the same package and taken to the laboratory frozen. Prior to bacteriological analysis, the tissue samples were decontaminated by three different treatments: hexadecylpyridinium (HPC) to 0.75%, sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) at 6% and NaOH at 4% according to standard methods (OIE, 2009) and inoculated on two slopes of solid, egg-based Lowenstein-Jensen (LJ) media with 0.5% pyruvate, and two slopes of Stonebrink media, which were incubated at 37°C and observed once weekly for 12 weeks.

DNA templates were extracted from colonies by suspension in 200 µl of distilled water for 10 min at 100°C. The isolated microorganisms were confirmed by m-PCR (Figueiredo et al., 2009) and identified by HPLC.

The HPLC was performed according to Furlanetto et al. (2014). A suspension of acid-fast bacteria grown in LJ medium was collected by a swab and saponified with 2 ml KOH 25% in methanol:H<sub>2</sub>O (v:v) autoclaved for 1 h at 121°C, 15 psi, to cleave the mycolic acids bound to the cell wall. Mycolic acids were then separated by acidification with HCl:H<sub>2</sub>O (v:v) and extraction into chloroform. After conversion to ultraviolet (UV)-absorbing *p*-bromophenacyl esters (Pircen<sup>®</sup>) and clarification with HCl:H<sub>2</sub>O:metanol (1:1:2, v:v:v), the mycolic acids were analyzed on a reverse-phase C18 100 x 4.6 mm column (Kromasil<sup>®</sup>) using high performance liquid chromatography. A methanol and dichloromethane (methylene chloride) gradient generated by microprocessor-controlled pumps was used to separate the mycolic acid esters, which were detected with a UV detector at 260 nm. Reproducible chromatographic patterns containing combinations of different diagnostic peaks were obtained by using reference strains (*M. abscessus* ATCC 19977, *M. africanum* ATCC 25420, *M. agri* ATCC 27406, *M. aichiense* ATCC 27280, *M. asiaticum* ATCC 25276, *M. aurum* ATCC 23366, *M. avium* ATCC 25291, *M. bovis* ATCC 19210, *M. bovis* BCG INCQS 00062, *M. chelonae* ATCC 35752, *M. flavescens* ATCC 14474, *M. fortuitum* ATCC 6841, *M. gastri* ATCC 15754, *M. godornae* ATCC 141470, *M. intracellulare* ATCC 13950, *M. malmoense* ATCC 29571, *M. mucogenicum* ATCC 49650, *M. scrofulaceum* ATCC 19981, *M. simiae* ATCC 25275, *M. terrae* ATCC 15755, *M. tuberculosis* ATCC 25177, *M. vaccae* ATCC 15483, *M. triviale* ATCC 23292). The chromatographic pattern for each strain was examined for differences in the heights for pairs of peaks. HPLC patterns were grouped according to species, and the values calculated for each ratio were combined, sorted in numerical order, and examined regarding their ability to discriminate species, using the range of the relative standard deviation (RSD) of the absolute retention times (ART) and the relative retention times (RRT).

### DNA preparation from tissues of CITT-reactive cows

DNA was extracted from the pooled samples (lymph nodes and lung), in order to obtain a representative aliquot of each animal,

based on a modification of a QIAamp Blood and Tissue Kit (Qiagen<sup>®</sup>) already described by Furlanetto et al. (2012). A small piece of tissue (approximately 1 g) was macerated and diluted and a aliquot of the 1 mL was taken. The pellet was suspended in 180 µl of lysis buffer (20 mg/mL lysozyme in 20 mM Tris-HCl, pH 8.0; 2 mM EDTA and 1.2% Triton) and incubated for 1 h at 37°C. After this step, DNA extraction followed the manufacturer's recommendations. DNA was quantified in a Nanodrop ND1000 (Thermo Scientific, USA).

### m-PCR assay

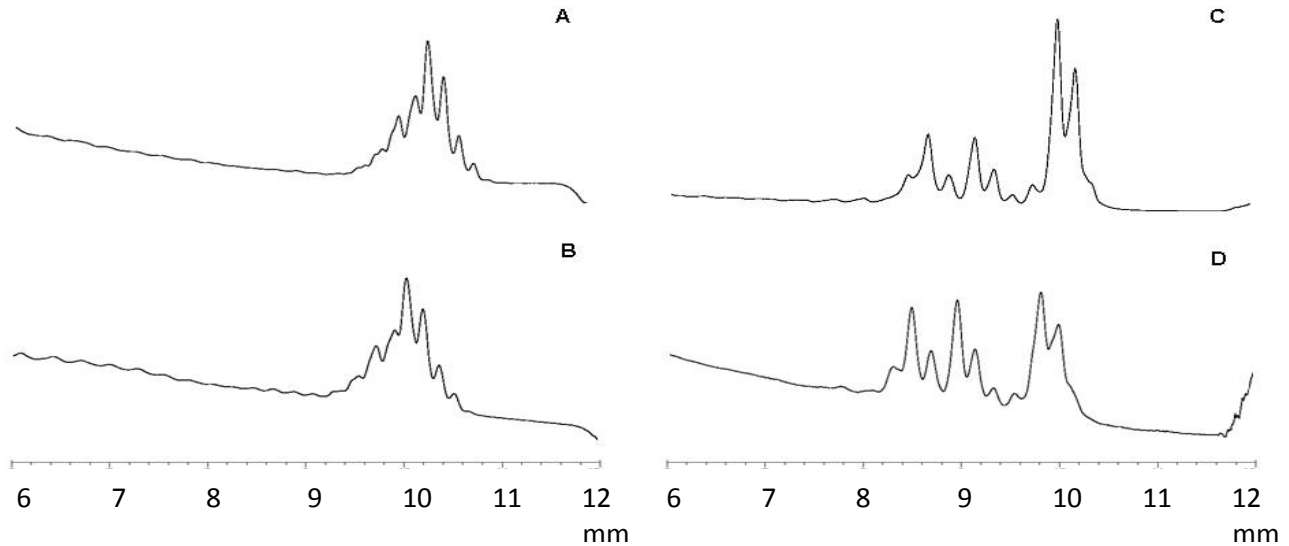
The m-PCR was performed according to Figueiredo et al. (2009). The reaction mix (50 µL) contained 5 µl of 10× PCR buffer (Invitrogen<sup>®</sup>), 200 µM dNTP (GE Healthcare<sup>®</sup>), 2.5 U of recombinant *Taq* polymerase (Invitrogen<sup>®</sup>), 2.0 mM MgCl<sub>2</sub>, 50 ng of each DNA template, and 0.2 µM of each primer JB21 (5'-TCGTCCGCTGATGCAAGTGC-3') and JB22 (5'-CGTGAACGTAGTCGCCTGC-3'), targeting the *RvD1-Rv2031c* sequence, specific for *M. bovis* and INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAGGCGTCGGTGACAAA-3'), targeting the *IS6110* region, specific for MTC. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems<sup>®</sup>) with the following cycling parameters: 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, with a final extension at 72°C for 7 min. PCR products were checked by electrophoresis on 1.5% agarose gels stained with ethidium bromide (10 µg/mL).

Clinical samples were considered positive when double bands of 500 (*M. bovis*) and 245 bp (MTC) DNA were observed. DNA templates from reference strains *M. avium* (ATCC 13950), *M. fortuitum* (ATCC 6841), *M. terrae* (ATCC 15755), *M. vaccae* (ATCC 15483), *M. xenopi* (ATCC 33501), *M. flavescens* (ATCC 14474) and *M. scrofulaceum* (ATCC 19981) were used as negative controls to control cross-contamination and confirm the specificity of the m-PCR assays.

## RESULTS AND DISCUSSION

From the 34 cows considered CITT-reactive, only nine animals presented macroscopic lesions compatible with granuloma in the lungs, were considered suggestive lesions. However, tissues from all 34 animals were collected, pooled and submitted to the culture and m-PCR assays. Seventeen (50%) of the samples were culture positive for *Mycobacterium* sp, where the presence of *M. bovis* was confirmed in 15/17 (88.2%) isolates, by m-PCR assays (Figueiredo et al., 2009) and HPLC analyses (Figure 1B). The others two isolated mycobacterium (m-PCR negative assay) were identified as *M. fortuitum* by HPLC analysis (Figure 1D). The totality of the remaining samples, 17, failed to grow in culture.

Decontamination with 0.75% HPC yielded *M. bovis* recovery from 10 samples, whereas 4% sodium hydroxide or 6% sulphuric acid yielded only recovered, *M. bovis* from six and five samples, respectively. The proportion of positive samples was higher for HPC than for each of the other two methods. When using both 0.75% HPC and 6% sulphuric acid methods for decontamination, it was possible to identify 13 of 15 (86, 6%) infected cows.



**Figure 1.** Representative reverse-phase HPLC chromatograms of mycolic acid methylesters from reference strains and isolated microorganisms. (A) Chromatogram from *M. bovis* ATCC 19210 (representative of MTC); (B) representative chromatogram of 15 *Mycobacterium bovis* (CITT-reactive and m-PCR positive), (C) chromatogram from *M. fortuitum* ATCC 6841; (D) representative chromatogram from two isolated *Mycobacterium fortuitum* (CITT-reactive and m-PCR negative).

It was possible to identify all isolates (17) by HPLC, while the m-PCR technique identified only *M. bovis* (15). HPLC was more efficient than m-PCR adopted here because the mycolic acids from the cell wall generate characteristic chromatograms of each species or group. On the other hand, this is a technique that requires more expensive equipment and expertise for deployment as a BTB routine testing (Figure 1).

Multiplex PCR tests of tissue samples from CITT-reactive cows were able to amplify the target DNA in 23/34 (67.6%) of the assayed samples (Figure 2). *M. bovis* by m-PCR assays were identified in 10 samples where no culture growth was observed, which means that 59% of negative-culturing samples came from infected cows.

PCR assays have been successfully applied to detect MTC and *M. bovis* from clinical cattle samples (Cardoso et al., 2009; Figueiredo et al., 2009). In the present study, the PCR test was sensitive enough to detect *M. bovis* in a large proportion (59%) of the samples that failed to grow in culture. This was also emphasized by Liébana et al. (1995) and Zanini et al. (2001). For Miller et al. (2002) and Araujo et al. (2005), the efficiency of the culture method used as a first criterion for *M. bovis* identification is low because of the small number and live bacilli presence in some tissues, because of a short delay in delivering the tissues to the laboratory or because of the sensitivity of the mycobacteria to sodium hydroxide used in the Petroff method.

For the remaining 11 CITT-reactive cows, where both culturing and m-PCR assays failed to identify *M. bovis*, it is possible that there was an inhibitory effect during the

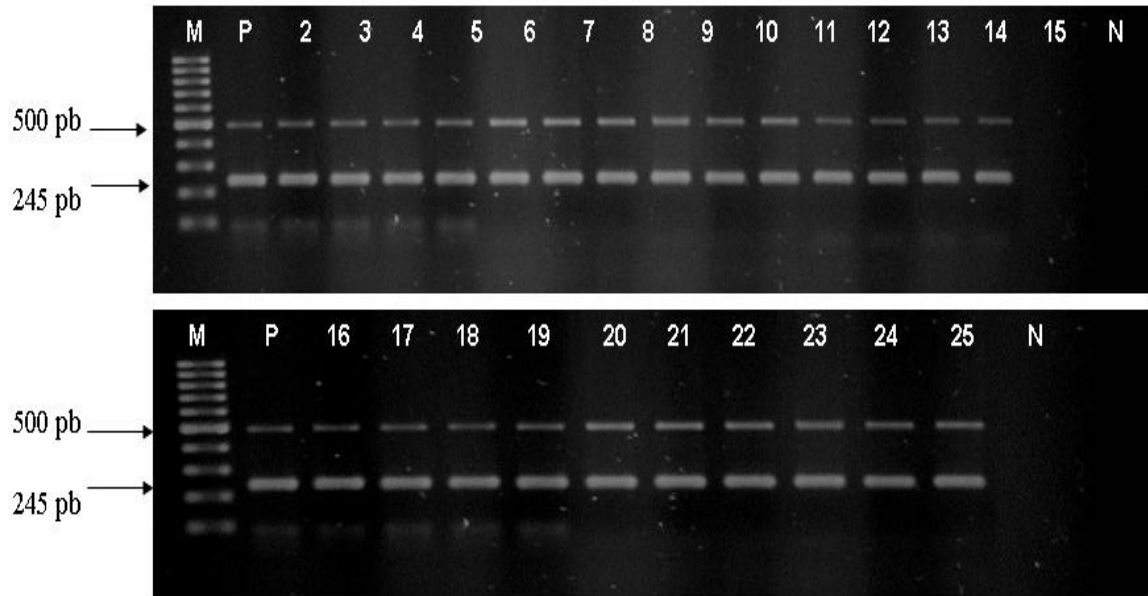
PCR assay (Al-Soud and Radstrom, 2001; Cardoso et al., 2009). Some authors (Zanini et al., 2001; Cardoso et al., 2009) also observed less than 100% sensitivity. PCR assays are not able to detect samples that contain a small numbers of pathogens, mainly in paucibacillary tissue samples. The 11 samples from CITT-reactive cows, not confirmed by culturing and m-PCR tests, probably presenting paucibacillary lesions (low amount of *M. bovis* bacillus), fit the characteristics of a recent intra-herd infection. It is generally accepted that the CITT is related to *M. bovis* infections and not necessarily to disease (Neill et al., 1994).

## Conclusions

Our results indicate that m-PCR is able to detect *M. bovis* DNA directly in tissue samples and represents a valid additional tool for the post mortem diagnosis of BTB. Multiplex PCR assay is faster and more specific than culture-based diagnosis in *M. bovis* detection and can reduce the diagnosis time from 90 days to approximately two days. Moreover, the m-PCR test is useful when the bacilli are non-viable and cannot be detected by culture methods, being a valuable aid during the sanitary inspection of slaughterhouses for the condemnation of carcasses that show suspected lesions of the bovine tuberculosis.

## Conflict of interests

The authors did not declare any conflict of interest.



**Figure 2.** Direct detection of *M. bovis* DNA in tissue samples from CITT-reactive animals. DNA templates obtained from tissue samples were tested by m-PCR. Amplicons were resolved on 1.5% agarose gels stained with ethidium bromide; lane M: 100 bp DNA ladder (Promega®); lane P, positive control *M. bovis* IP; lane N, negative control (without mycobacterial DNA); lane 2-25, 500 bp (*M. bovis*) and 245 bp (MTC) amplified fragments from tissue samples from CITT-reactive cattle; lane 15, negative control with DNA pool of *M. avium* (ATCC 13950), *M. fortuitum* (ATCC 6841), *M. terrae* (ATCC 15755), *M. vaccae* (ATCC 15483), *M. xenopi* (ATCC 33501), *M. flavescens* (ATCC 14474) and *M. scrofulaceum* (ATCC 19981). Three independent PCR tests were performed for each cow, using the same template DNA.

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