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# Detection of *Mycobacterium* sp. by multiplex PCR directly from suspicious granulomas from cold chambers in the state of Bahia, Brazil

Evelin Santos[1] https://orcid.org/0000-0002-0149-2890
Italaney Fehlberg[1] https://orcid.org/0000-0001-6570-498X
Bruno Fernandes[1,*] https://orcid.org/0000-0002-4465-6402
Adriano de Alcântara[1] https://orcid.org/0000-0003-0115-4987
Bianca Silva[1] https://orcid.org/0000-0002-7013-4181
Robson Cerqueira[1] https://orcid.org/0000-0001-5054-0353

1. Universidade Federal do Recôncavo da Bahia – Laboratório de Imunologia e Biologia Molecular – Cruz das Almas (BA), Brazil.
*Corresponding author: brunofernandesnv@gmail.com

**ABSTRACT**

The diagnosis of bovine tuberculosis (TB) by molecular techniques has been broadly studied. These methods allow accelerating the diagnosis, in addition to presenting high specificity and sensitivity in the identification of the pathogen, critical characteristic for public health, especially when it comes to the direct diagnosis of the biologic samples, which has been little explored. This paper has evaluated a multiplex polymerase chain reaction (mPCR) as a tool to diagnose TB, which was performed directly on the granulomatous material of suspicious lesions collected in a cold chamber under state inspection in the state of Bahia, Brazil. Of the 74 samples evaluated, 14.86% were positive, with 10.81% positive for mPCR and culture, 4.05% negative for cultivation and positive for mPCR. The correlation between the cultivation and the mPCR presented agreeance higher than 61.54% of the cases. The results have indicated that the protocol proved itself effective, fast and very promising in the surveillance in slaughterhouses for the diagnosis of tuberculosis directly from the granuloma.

**Keywords:** multiplex PCR; tuberculosis; granuloma; *Mycobacterium bovis*.

## INTRODUCTION

Among the main epidemiologically relevant species of the genus *Mycobacterium*, those belonging to the *Mycobacterium tuberculosis* complex (MTC) stand out: *Mycobacterium bovis*, *M. tuberculosis*, *Mycobacterium bovis BCG*, *Mycobacterium africanum*, *Mycobacterium caprae*, *Mycobacterium pinnipedii*, *Mycobacterium canettii*, *Mycobacterium microti* (PRODINGER et al., 2005; SKORIC et al., 2007) and the newest member, *Mycobacterium mungi* (ALEXANDER et al., 2010). This group is responsible for most tuberculosis cases (TB), both in humans and animals (MURAKAMI et al., 2009). Therefore, man can contract them and transmit them to animals (TORRES-GONZALEZ et al., 2013; VAN INGEN et al., 2012). In bovines, the disease is caused by *M. bovis*, which generates big economic damages (MÜLLER et al., 2013) and risks to the health of the human population consuming animal products, considering that the main species of MTC are resistant to boiling (TEWART et al., 2013; VAN DER MERWE et al., 2009). PACHECO et al. (2007) describe that the most reliable methods of diagnosis are the ones performed directly. Due to the difficulties found in the diagnosis of bovine TB, such as the limitations of tuberculin and serum tests and the long time demanded the identification of the agent in the bacterial methods, the interest for molecular tests has increased. The same way as the polymerase chain reaction (PCR), especially for detecting of the agent in the clinical samples, stresses two relevant points: the correct choice for the primers and the quality of the

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DNA extraction technique (OLIVEIRA et al., 2007). Proved by HALSE et al. (2011), studies in the difference regions of the bacterial genomes have demonstrated that the presence or absence of these regions may be useful in telling apart the members of this complex. Though some tests for humans have already been described, this methodology for diagnosis has still been little explored in animals (COSTA et al., 2013; UEYAMA et al., 2014). Among the advantages of such a technique are the reduction of time to achieve the diagnostic result, detection of several species in a single reaction, independence of bacterial cultivation and the viability of the microorganism for its detection, and also the possibility of diagnosing samples conserved for longer periods (COSTA et al., 2013; FURLANETTO et al., 2012a. PARRA et al. (2008) and ARAÚJO (2014) claim that it is necessary to develop diagnosis systems directly applicable to biological samples collected directly from the slaughterhouse. Molecular tests, such as described by WARREN et al. (2006), who has used a multiplex PCR (mPCR) to identify species of genus *Mycobacterium*, have become interesting for the inspection in routine labs, being adapted and tested through cultivation (ALZAMORA FILHO et al., 2014). Thus, this study aimed to evaluate an mPCR as a tool for diagnosing TB performed directly in granulomatous material from suspicious bovine lesions arising from slaughterhouses cold chambers under state inspection in the regions of Recôncavo Baiano and the southwestern Bahia, Brazil.

# MATERIAL AND METHODS

## Local and clinical specimen

The study was carried out in Bahia in two slaughterhouse cold chambers under state inspection, both important places for performing inspected slaughters. A total of 91,473 bovines from 226 municipalities from Bahia were slaughtered and underwent a systematic assessment of carcasses and viscera by sanitary inspection. During the exam, a search for suggestive TB lesions was performed within the guidelines for inspection according to the Regulation for Industrial and Sanitary Inspection of Animal Products. When suspicious, the sanitary decision was made pursuant to article 196 of the same regulation, proceeding with total condemnation or partial rejection of the carcasses (BRAZIL, 1997). A total of 74 samples of granulomas were collected between March 2012 and January 2014 at slaughterhouses with state inspection, located in Recôncavo Baiano and the Southwestern Bahia. The samples were collected considering regions presenting nodular lesions varying in size and format containing purulent, caseous or calcified exudate (in this case lungs, liver and lymph nodes, Fig.1), from healthy bovines at the antemortem examination and were considered suggestive of TB, without distinction of age, race, gender or region of origin. The fragments with an approximate size of 2×2 cm were collected with a sterile universal collector and stored at –20 °C, from 3 to 12 months. Then, they were sent to the Infectious Diseases Laboratory (LDI) of the Federal University of Recôncavo da Bahia (UFRB) for the performance of an mPCR; duplicates of the samples were made and sent to the Laboratory of Bacterial Zoonoses (LBZ) from the University of São Paulo (USP) for cultivation and identification. The LBZ is a reference laboratory in Brazil for diagnosing bovine TB using traditional and molecular techniques.

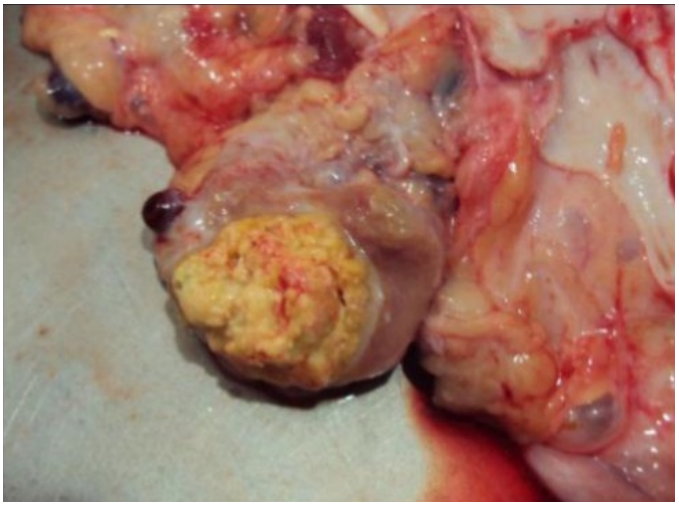

**Figure 1.** A representative lesion in the thoracic lymph node from an animal with suggestive TB injury. Note the granulomatous mass with yellowish color and doughy consistency.
Source: Elaborated by the authors.

## Cultivation

The samples were decontaminated with hexadecyl pyridinium chloride (HPC) at 1.5% for 30 min with subsequent sowing in Löwenstein–Jensen and Stonebrink–Leslie media and incubation at 37 °C. The tubes were observed weekly for 60 days to follow up on the bacterial growth. The samples that presented characteristic genus growth were identified by PCR. DNA extraction was conducted directly from the granuloma. The extraction protocol was adapted for the first time for bovines, aiming at the lysing of *Mycobacterium* sp.'s cell wall, using QIAamp DNA Mini Kit (QIAGEN, Brazil) according to the manufacturer's instructions. Modifications in the time of action of the lysozyme and the proteinase K were carried out according to FEHLBERG (2010). Briefly, 25 mg of the center of the content of the granulomas were macerated and resuspended in 180 µL of solution (20 mg·mL$^{-1}$ lysozyme; 20 mol·L$^{-1}$ Tris-HCl, pH 8.0; 2 mmol·L$^{-1}$ EDTA; 1.2% triton X-100) containing 3.6 mg of lysozyme for each 180 µL of definitive solution; and incubated for 2 h at 37 °C. Then, 20 µL of proteinase K solution is 600 mAU·mL$^{-1}$ solution (or 40 mAU·mg$^{-1}$ protein) (QIAGEN, Brazil) and 200 µL lyse AL buffer (QIAGEN, Brazil) were added, followed by a 1-h incubation at 56 °C and, right afterward, 15 min at 95 °C for the inactivation of the proteinase K, and washed. DNAs were eluted with 50 µL of AE buffer (10 mol·L$^{-1}$ Tris-CL; 0.5 mol·L$^{-1}$ EDTA; pH 9.0), and the concentrations and purity rate were determined by a nanodrop spectrophotometer Thermo Scientific 2000c. The concentration of the samples was adjusted to 25 ηg·µL$^{-1}$ and stored at –20 °C the mPCRs were carried out.

## Primers

The sequence of the primers used for identifying and differentiating the five species (*M. tuberculosis*, *M. bovis*, *M. canettii*, *M. microti* and *M. bovis BCG*) of the *Mycobacterium* complex were obtained from the paper published by WARREN et al. (2006). According to WARREN et al. (2006), the test conditions occurred, and the enzyme used was the HotStarTaq plus DNA polymerase (QIAGEN, Brazil). As positive controls, DNAs of *M. bovis*, *M. tuberculosis*, *M. avium*, and *M. kansasii* were gently granted by the Laboratory of Immunology and Molecular Biology of Federal University of Bahia, Brazil. DNA extraction and amplification were performed in physically separated areas to minimize the risks of contamination. The electrophoresis (6 V·cm–1) of the amplified products occurred in 3% (w·v$^{-1}$) agarose gel submerged in TBE 1X buffer and using the colorant Blue Green Loading Dye I (LGC Biotecnologia, Brazil).

## Statistical analysis

To evaluate the results, the generalized linear model (GLM) was used for a binomial distribution, ANOVA, logit connection function, having the averages evaluated by Bonferroni's average test at 5% significance. The program selected was the R/2011. To determine the sensitivity and relative specificity of the mPCR tests, the formula described by MATHIAS et al. (1998) (Eqs. 1 and 2) was used. As a standard gold test, culture detection of positive samples was considered.

$$\text{Sensibility} = \frac{\text{Positive samples detected by the mPCR test} \times 100}{\text{Total positive samples by gold standard method (culture)}} \quad (1)$$

$$\text{Specificity} \quad \frac{\text{Negative samples detected by the mPCR test} \times 100}{\text{Total negative samples by gold standard method (culture)}} \quad (2)$$

# RESULTS

Considering the 74 samples analyzed, the percentage of positivity in isolation was 14.86% (11/74). Among them, 10.81% (8/74) positive samples were positive in mPCR and culture, and 4.05% (3/74) were negative in culture and positive in mPCR (Table 1). The crops identified present characteristic growth for *M. bovis* in Stonebrink–Leslie medium, confirmed by PCR through isolated colonies. No growth in Löwenstein–Jensen medium was observed for any of the samples cultivated. In the molecular analysis, the concentrations of the DNAs extracted directed from the granulomas varied between 115.2 and 6,404.1 ng·µL$^{-1}$, and they all presented an adequate level of purity. An amplification profile characteristic for *M. bovis* was observed in 24.3% of the samples (Fig. 2.) The sensibility of mPCR was calculated considering eight positive samples by mPCR among 11 true positive samples by culture; therefore, the sensibility of the mPCR test was 72.73%. Equally, the specificity was 94.64% (53/56). A statistical correlation was performed between the two techniques and a concordance of 61.54% between the cultivation and mPCR.

**Table 1.** Relationship between cultivation and mPCR from granulomatous material suspicious lesions for diagnosis of bovine TB in slaughterhouses of the regions of Recôncavo Baiano and the southwestern Bahia, Brazil.

| Slaughterhouses/ Cultivation | mPCR | | Total |
|---|---|---|---|
| | POS | NEG | |
| POS | 8 (10.81%) | 03 (4.05%) | 11 (14.86%) |
| NEG | 10 (13.51%) | 53 (71.62%) | 63 (85.14%) |
| TOTAL | 18 (24.32%) | 56 (75.68%) | 74 (100%) |

Source: Elaborated by the authors.

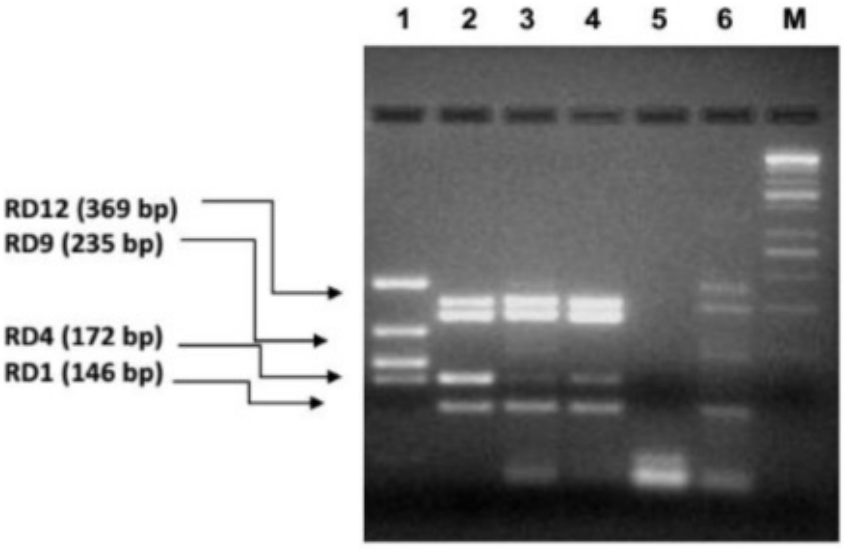


**Figure 2.** Amplification profiles of the samples collected directly from the material of bovine abscesses. Lane 1, positive control for *Mycobacterium tuberculosis*; lane 2, positive control for *Mycobacterium bovis*; lanes 3 and 4, positive samples; lane 5, negative control; lane 6, negative sample and lane M, molecular marker (100 pb ladder).
Source: Elaborated by the authors.

## DISCUSSION

In the control of bovine TB, surveillance in cold chamber slaughterhouses is an important tool. Data collections performed in cold chambers for bovines demonstrate the differences in the rates of lesions suspected with TB (ALZAMORA FILHO et al., 2014; COSTA, 2012). The justifications for such contrast are various, and the sensitivity of the inspection and the breeding system must also be considered. This surveillance occurs through observation and collection of suspicious samples, being subsequently investigated through cultivation, and molecular detection can take months for confirmation can take months.

PARRA et al. (2008), SANTOS et al. (2010), and CORNER et al. (2012) defend that, due to the fact of the number of viable bacteria being small in some tissues, the efficiency of cultivation, as the main method for the identification of the *M. bovis* must be discussed. They also consider, with valid arguments, the interval of time from the collection of samples to their arrival at the laboratory; sensitivity of the bacilli to the process of decontamination; difficulty in growing in cultivation media; death of the *Mycobacterium* after the production of the granuloma; and the lesion being caused by others infectious agent. CORNER (1994) affirms that, even after the storage for one year at –20°C, 78% of the positive samples remain detectable through microbiological examination, which was reaffirmed by this paper. The reported difficulties encountered in diagnosing TB using caseous material pointed to the use of molecular techniques to overcome such problems of molecular techniques for the diagnosis of TB, especially in the detection through the caseous material. The efficiency of these tests depends on several factors, such as the quality of the DNA extracted, low levels of contaminants, the correct choice of the primers for amplification, as well as the application of adequate protocols for the extraction of the nucleic acids, especially for paucibacillary samples (RIBEIRO, 2006; SALAZAR, 2005; TAYLOR et al., 2007).

In the process of nucleic acid extraction, one of the key steps is cellular lysis, which, if not optimized, will not extract the DNA of the microorganisms in sufficient quantity, which becomes more difficult in the case of mycobacteria for their

thick layer of mycolic acids and reduced quantity in the sample arising from the granuloma. The procedure for extraction used for this paper, based on FEHLBERG (2010), allowed the extraction of the genetic material in adequate quantity and purity similar to what was cited in the paper. The impact of different extraction techniques on the diagnosis for TB is widely discussed and calls attention to the steps of collection and extraction as essential for an accurate diagnosis (BARANDIARAN et al., 2013; NAKATANI et al., 2004; TAYLOR et al., 2007; THACKER et al., 2011). The results obtained in this study show the extraction process seems to be adequate for the success of the diagnosis. Inhibitory factors are other significant inconveniences that may make amplification difficult (ARAÚJO, 2014; BARANDIARAN et al., 2013); however, a high sensibility was achieved in the present study suggesting the sample preparations in this study had a low inhibitory factor for the mPCR.

In a study carried out by PARRA et al. (2008) with 125 samples of bovine tissues, no positivity in negative samples during real-time PCR negative samples in culture. However, there were positive samples in the culture and not detected by the real-time PCR. A total of 111/125 was positive in cultivation, and 82/125 (65.6%) were positive on real-time PCR. The samples positive during the PCR may be explained by the unviability of the pathogen for isolation but with genetic material preserved for detection by the molecular technique. Unviability may arise from the aggressive process of decontamination performed for the cultivation to be carried out, resulting in the bacilli's death. Contamination of samples is another problem that makes mycobacterial growth more difficult (GORMLEY et al., 2014). The results of the bacterial tests may also be affected by the growth and incubation conditions used, as well as the restricted distribution of mycobacteria in the tissues (CORNER et al., 2012) positive samples found in isolates may be due to contamination.

A study performed in Rio de Janeiro with 24 bovines presenting TB history, slaughtered and necropsied, resulted in 78.3% positivity while analyzed by an mPCR, against 83% positive results during collection (ZARDEN et al., 2013). These results are superior to those presented in this paper, considering the performance of the PCR from isolates.

There are reports of protocols successfully diagnosing tuberculosis from caseous material (CARDOSO et al., 2009; FURLANETTO et al., 2012b) stressing, within this context, the association of the methods and the establishment of the correlation with the gold standard methods pattern that are still vital. Protocols demonstrated higher sensitivity and specificity than found during this paper; however, these approaches involved nested PCR with more experimental steps and the possibility of higher rates of contamination (ARAÚJO, 2014; COSTA et al., 2013; THACKER et al., 2011). FURLANETTO et al. (2012b) have used three methods of diagnosis for samples from cold chambers and have demonstrated that the mPCR (7.5% positive results for *M. bovis*) presented different performance against, which has presented 1.5% isolation. CARDOSO et al. (2009) analyzed 35 lymph node samples in Paraná, collected from bovines with macroscopic TB lesions during the inspection in a slaughterhouse and related a frequency of positive results during PCR similar to the results of the positive cultivation (51.5% versus 54.5%). MARASSI et al. (2013) suggest that none has made it possible to identify all the animals infected among the existing methods. CEZAR et al. (2016), studying qPCR for direct detection of *M. bovis* in milk and blood samples of cattle from Pernambuco, Brazil, demonstrated the effectiveness of technical and detectable *M. bovis* DNA in one milk sample what may pose a risk to public health.

In conclusion, the mPCR test has demonstrated its ability to detect the pathogen causing bovine TB directly from lesions suggesting the disease and make it possible to detect other species of mycobacteria important for the epidemiologic surveillance.

## AUTHORS' CONTRIBUTIONS

**Conceptualization:** Bahia, R.C. **Data curation:** Santos, E.S.V.; Fernandes, B.P.; Fehlberg, I.; Alcântara, A.C.; Silva, B.P. **Formal analysis:** Santos, E.S.V.; Fernandes, B.P.; Fehlberg, I.; Alcântara, A.C.; Silva, B.P.

## AVAILABILITY OF DATA AND MATERIAL

All data generated or analyzed during this study are included in this published article.

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## CONFLICTS OF INTEREST

The authors declare there is no conflict of interests.

## ETHICAL APPROVAL

Not applicable.

## ACKNOWLEDGEMENTS

Not applicable.

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