Detection of Mycobacterium Sp. by PCR Multiplex Directly from Suspicous Granulomas from Cold Chambers in the State of Bahia, Brazil

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Abstract

The diagnosis for bovine tuberculosis (TB) by molecular techniques has been studied broadly. 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 PCR as a tool to diagnose tuberculosis, which was performed directly on the granulomatous material of suspicious lesions collected in cold chamber under State inspection in the State of Bahia, Brazil. Among the samples collected recently, 61.54% of them have shown positive results during isolation and 54% have shown M. bovis profile in the mPCR. These samples with longer time of storage 4.92% and 18% were positive during isolation and molecular technique, respectively. The correlation between the cultivation and the mPCR has presented agreement 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: PCR Multiplex; Tuberculosis; Granuloma; Mycobacterium bovis

Introduction

Among the main epidemiologically relevant species of the genus Mycobacterium, those belonging to the M. tuberculosis (MTC) stand out: M. bovis, M. tuberculosis, M. bovis BCG, M. africanum, M. caprae, M. pinnipedii, M. canettii, M. microti [1,2] and the newest member, M. mungi [3]. This group is responsible for most cases of TB, both in humans and in animals [4]. Therefore, man can contract them from as well as transmit them to animals [5,6]. In bovines, it is a disease caused by M. bovis which generates big economic damages [7] and risks to the health of the human population consuming animal products, considering that the main species of MTC are resistant to boiling [8,9]. Pacheco [10] describe that the most reliable methods of diagnosis are the ones performed directly. However, the difficulty in obtaining in vivo samples makes it difficult to use these methods in a regular basis.

Due to the difficulties found in the diagnosis of bovine TB, such as the limitations of allergy and serum tests and the long time demanded for 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 the detection of the agent in the clinical samples, stressing two relevant points: the correct choice for the primers and the quality of the DNA extraction technique [11]. Proved by Hake [12], studies in the difference regions (DR) 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, in animals, this methodology for diagnosis has still been little explored [13,14]. Among the advantages of such 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 [14,15].

Parra & Araújo [16,17] claim it is necessary to develop diagnosis systems that are directly applicable to biological samples collected directly from the slaughterhouse. Molecular tests, such as Warren [18], who has used a multiplex PCR to identify species of genus Mycobacterium, have become interesting for the inspection in routine labs, being adapted and tested through cultivation [19]. Thus, the aim of this study was to evaluate an mPCR with tools...
for the diagnosis of tuberculosis performed directly in granulomatous material from suspicious bovine lesions arising from slaughterhouses - cold chambers under state inspection in the regions of Recôncavo and Southwestern Bahia, Brazil.

**Materials 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 tuberculosis lesions was performed within the guidelines for inspection according to the Regulation for Industrial and Sanitary Inspection of Animal Products (RISPOA). 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 [20].

![Figure 1: Representative lesion in the thoracic lymph node from an animal with suggestive tuberculosis injury. Note the granulomatous mass with yellowish color and doughy consistency.](image)

A total of 74 samples of granulomas were collected between March 2012 and January 2014. Out of these, 61 samples were obtained between March and November 2012, conserved for a long period; and 13 were collected in the period between October 2013 and January 2014. Regions presenting nodular lesions varying in size and format containing purulent, caseous or calcified exudate (in this case - lungs, liver and lymph nodes) - Figure 1, from healthy bovines at the ante mortem examination were considered suggestive of tuberculosis, without distinction of age, race, gender or region of origin. The fragments with approximate size of 2x2cm were the collected with a sterile universal collector and stored at -20 °C. The, they were forwarded to the Laboratory of Infectious Diseases (Laboratório de Doenças Infecciosas - LDI) of Universidade Federal do Recôncavo da Bahia (UFRB) for the performance of a mPCR and one aliquot of each sample was sent to the Laboratory of Bacterial Zoonosis (Laboratório de Zoonoses Bacterianas) of Universidade de São Paulo (USP) for cultivation.

**Cultivation**

The samples were decontaminated with HPC (Hexadecylpyridinium Chloride) at 1.5% for 30 minutes with subsequent sowing in Lowenstein-Jensen and Stonebrink Lesle media and incubation at 37 °C. The tubes were observed weekly during 60 days, to follow-up the bacterial growth. The samples that presented characteristic genus growth were identified by PCR.

**DNA Extraction Directly from the Granuloma**

The protocol of extraction 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 protease K were carried out according to Fehlberg [21]. Briefly, 5 mg of the center of the content of the granulomas were re-suspended in 180µl of solution (20mg/ml lysozyme; 20mM 3 Tris-HCl, pH 8.0; 2mM EDTA; 1.2% Triton X-100) containing 3.6mg of lysozyme 31 for each 180µl of definitive solution; and incubated for 2h at 37 °C. Then, 20 µl of 32 protease K solution is 600 mAU/ml solution (or 40 mAU/mg protein) (QIAGEN, Brazil) and 200µl lyse AL buffer (QIAGEN, Brazil) were added, followed by a 1-hour incubation at 56 °C and, right afterwards, 15 minutes at 95 °C for the inactivation of the protease K. The DNAs were eluded into 50µl of AE buffer (10mM Tris-Cl; 0.5mM EDTA; pH 9.0) and the concentrations and purity rate were determined by a nanodrop spectrophotometer Thermo Scientific 2000c. Right afterwards, they were standardized in 25ng and stored at -20 °C until the mPCRs were carried out.

**Primers**

The primers used for identifying and telling apart the five species (M. tuberculosis, M. bovis, M. Canetti, M microti and M. bovis BCG) of the Mycobacterium complex were obtained from the paper published by Warren [18].

**Multiplex Polymerase Chain Reaction (mPCR)**

The test conditions occurred according to Warren [18] and the enzyme used was the HotStarTaq plus DNA polymerase (QIAGEN, Brazil). The positive controls, M. bovis and M. tuberculosis DNA of M. avium and M. kansasii were gently granted by the Laboratory of Immunology and Molecular Biology of Universidade Federal da Bahia, Brazil. The steps of the PCR were carried out in physically separated areas in order to minimize the risks of contamination. The electrophoresis (6V/cm) of the amplified products occurred in agarose gel at 3% (w/v) submerged in TBE 1X buffer and using the colorant Blue Green Loading Dye I (LGC Biotecnologia, Brazil).

**Statistical Analysis**

In order 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 15 described by Mathias [22] as represented be-
low was used. As gold 16 standard test, culture detection of positive samples was considered.

\[
\text{Sensibility} = \frac{\text{Positive samples detected by the mPCR test } X}{\text{Total positive samples by gold standard method (culture)}}
\]

\[
\text{Specificity} = \frac{\text{Negative samples by mPCR test } X}{\text{Total negative samples by gold standard method (culture)}}
\]

Results

![Amplification profile of the cells collected directly from the material of bovine abscesses. Line 1, positive control for *M. bovis*; lines 3 and 4, positive samples; line 5, negative control; line 6, negative sample and line M, molecular marker (100pb ladder).](image)

Considering the 74 samples analyzed, the percentage of positivity in isolation was 14.86% (11/74). Among the 13 samples with maximum conservation time of 3 months and the 61 with over one-year conservation submitted to the bacterial examination, 61.5% (8/13) and 4.92% (3/61), respectively, present characteristic growth for *Mycobacterium bovis* in Stonebrink-Leslie medium, being confirmed by PCR through the colonies isolated. No growth in Lowenstein-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 115.2ng/µL and 6,404.1 ng/µL and they all presented an adequate level of purity. An amplification profile characteristic for *M. bovis* was observed in 24.3% of the samples (Figure 2). The characteristic profile for the other species of micro bacteria researched. The sensitivity of mPCR was calculated considering 8 positive samples by mPCR 38 among 11 true positive samples by culture, therefore the sensibility of the mPCR 39 test was 72.73%. Equally the specificity was 94.64 % (53/56). A statistic correlation was performed between the two techniques and a 41 concordance of 61.54% between the cultivation and multiplex PCR.

Discussion

In the control of the bovine TB, the 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 [19,23]. The justifications for such contrast are various and the sensitivity of the inspection and the system of breeding must also be taken into consideration. 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, Santos & Corner [14,16,24] 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 other infectious agent.

Corner [25] 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 the diagnosis of 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 [26-28]. 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 [21], 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 tuberculosis is widely discussed and calls the attention for the steps of collection and extraction as essential for an accurate diagnosis [28-31]. The detection achieved in this study; the extraction process seems to be adequate for the success of the diagnosis. Inhibitory factors are other significant inconvenient that may make amplification difficult [17,31], however, in the present study a high sensibility was achieved suggesting the sample preparations in this study had low inhibitory factor for the mPCR.

In a study carried out by Parra [16] through 125 samples of bovine tissues, no positivity in negative samples during Real-Time PCR negative samples in culture. However, there was positive samples the culture and not detected by the Real-Time PCR. It was found 111/125 positive in cultivation, and of this total 82/125 PCR negative samples in culture. However, there was positive samples the culture and not detected by the Real-Time PCR. It was found 111/125 positive in cultivation, and of this total 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, which results in the death of the bacilli. Contamination of samples is another problem that makes micro bacterial growth more difficult [32]. 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. In a study carried out by Parra [16] through 125 samples of bovine tissues, there was no positive sample found in real time PCR negative cultures. However, samples were positive in culture and not detected by Real Time PCR. It was found 111/125 positive in cultivation, and of this total 82/125 (65.6%) were positive on Real-Time PCR. The samples negative during the PCR may be explained by absence of genetic material indispensable for detection by molecular technique or unviability may arise from the aggressive process of decontamination performed for the cultivation to be carried out, which results in the death of the bacilli positive samples found in isolates may be due to contamination of samples is another problem that makes micro bacterial growth more difficult. 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 [24].

A study performed in Rio de Janeiro with 24 bovines presenting TB history, slaughtered and necropsied, resulted in 78.3% positivity while analyzed by a multiplex PCR through the colony, against 83% positive results during collection [33]. These results are superior to the ones presented in this paper considering the performance of the PCR from isolates. There are reports of protocols successfully diagnosing tuberculosis from caseous material [34,35] stressing, within this context, the association of the methods and the establishment of the correlation with the gold 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 possibility of higher rates of contamination.

Furlaneto [35] has used 3 methods of diagnosis for samples from cold chambers and has demonstrated that the mPCR (7.5% positive results for M. bovis) was superior to the one presented in this paper, which has presented 1.5% isolation. Cardoso [34] analyzed 35 lymph node samples in Paraná, which were collected from bovines with macroscopic tuberculosis lesions during the inspection in a slaughterhouse and related a frequency of positive results during PCR similar to the results of the positive cultivation (5.1% versus 54.5%). Marassi [36] suggest that among the existing methods, none has made it possible to identify all the animals infected.

Cezar [37] studying qPCR for direct detection of Mycobacterium bovis in 38 milk and blood samples of cattle from the state of Pernambuco, Brazil, 39 demonstrated effectiveness of technical and detectable M. bovis DNA in one milk 40 sample what may pose a risk to public health [38-40]. In conclusion, the mPCR test has demonstrated its ability to detect the pathogen 42 causing bovine tuberculosis directly from lesions suggesting the disease, in 43 addition to making it possible to detect other species of mycobacteria important for 44 the epidemiologic surveillance.

Conflict of Interest
The authors declare there is no conflict of interests.

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