ORIGINAL ARTICLE

ELISA BASED ON RECOMBINANT MPB70 AND P27 FOR DETECTION OF ANTIBODIES AGAINST *Mycobacterium bovis*

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ABSTRACT

Bovine tuberculosis is a major disease caused by the bacterium *Mycobacterium bovis*. Skin tests and slaughter policies have reduced the incidence of bovine tuberculosis in many countries. However, more practical and efficient tools with high sensitivity and specificity values are needed. The aim of the present study was to develop an ELISA using the recombinant proteins MPB70 and p27 from *M. bovis* in order to detect antibodies against this bacterium in cattle. Sensitivity and specificity were respectively 88.7% and 94.6% for ELISA-MPB70 and 98.1% and 91.9% for ELISA-p27. The use of a serological test such as ELISA with recombinant MPB70 and p27, together with cell tests, may solve some of the problems regarding the diagnosis of bovine tuberculosis, such as inconclusive results and the lack of detection in anergic animals in advanced stages of the disease.

KEY WORDS: MPB70. p27. *Mycobacterium bovis.* Bovine tuberculosis.

RESUMO

ELISA baseado em MPB70 e p27 recombinantes para detecção de anticorpos contra *Mycobacterium bovis*

A tuberculose bovina é uma importante enfermidade causada pela bactéria *Mycobacterium bovis*. Testes de tuberculinização intradérmica e abate de animais infectados levaram à redução

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da incidência da tuberculose bovina em muitos países. Entretanto, são necessários métodos mais práticos e eficientes com maior sensibilidade e especificidade. O objetivo do presente estudo foi desenvolver um teste imunoenzimático (ELISA), utilizando as proteínas recombinantes MPB70 e p27 de *M. bovis*, que possibilitasse detectar anticorpos contra esta bactéria em bovinos. A sensibilidade e especificidade observadas foram, respectivamente, de 88,7% e 94,6% para o ELISA-MPB70 e de 98,1% e 91,9% para o ELISA-p27. O uso de testes sorológicos, como o ELISA com MPB70 e p27 recombinantes, juntamente com testes celulares, pode resolver alguns problemas relacionados ao diagnóstico da tuberculose bovina tais como os resultados inconclusivos e a ausência de detecção de animais anérgicos em estágios avançados da infecção.

DESCRITORES: Antígenos recombinantes MPB70 e p27. *Mycobacterium bovis*. Tuberculose bovina.

INTRODUCTION

Bovine tuberculosis is a major infectious disease caused by the intracellular bacterium *Mycobacterium bovis* (O'Reilly & Daborn, 1995). This disease accounts for important economic losses and is a major public health concern (Amadori et al., 2002).

In Brazil, the last data of official notification of bovine tuberculosis, from 1989 to 1998, showed an average prevalence of 1.3% of infected cattle. In a study conducted in Minas Gerais in 1999, a prevalence of 0.8% was found (Brasil, 2004). Recently, in the state of Paraíba, a prevalence of 0.25% of infected cattle was found (Figueiredo et al., 2010).

In this country, the control of bovine tuberculosis is based on the identification of infected animals using skin tests with purified protein derivative (PPD) and culling (Brasil, 2004). However, these methods show limitations, as cross-reactions with atypical mycobacteria may affect the specificity of these tests. Moreover, animals must be skin tested again after only 60 days since the first inoculation (Aagaard et al., 2006). Another limitation of skin tests regards false-negative reactions in chronically infected anergic animals (Monaghan et al., 1994), which go undetected and therefore become a source of infection in herds. These constraints have a negative impact on the Brazilian eradication program of tuberculosis. Thus, alternative immunodiagnostic methods are needed to improve the identification of animals infected with *M. bovis*.

As in other species of mycobacteria, the immune response first detected is the cellular type, followed by the subsequent production of specific antibodies. Thus, serological tests may complement the diagnosis of bovine tuberculosis (Plackett et al., 1989; Costa et al., 2011). Furthermore, serological tests may elucidate epidemiological cases of the disease in herds for which skins tests are inconclusive (Amadori et al., 1998).

The first standardizations of enzyme-linked immunosorbent assays (ELISA) for the diagnosis of bovine tuberculosis were performed using crude antigen extracts or PPD, which exhibited problems regarding cross reactions

with the *avium/intracellulare* mycobacterium group as well as a low power of discrimination between infected and non-infected animals (Amadori et al., 1998). Thus, a large number of studies currently focus on the identification of specific antigens for the *Mycobacterium tuberculosis* complex related to humoral response.

The present study demonstrates the use of ELISA with recombinant MPB70 and p27 proteins, which have proven to be specific for the *Mycobacterium tuberculosis* complex, for detection of total IgG against *M. bovis*.

MATERIALS AND METHODS

Cloning and expression of target genes

Primers were designed to amplify the *mpb70* and *p27* genes using polymerase chain reaction. The sequences of each primer pairs were:

mpb70F: 5'GTAAAGAACACAATTGCGGCAACCAGTTTC3'
mpb70R: 5'CCGGAGGCATTAGCACGCTGTCAA3'
p27F: 5'ATCAACTCCACCCGGATATATCT3'
p27R: 5'ATGTTCCTTGTATGAAAACGTGG3'

PCR reaction were prepared with 20 mM Tris pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 250 mM each dNTP, 100 ng each primer, 0.2 U *Taq* DNA polymerase (Invitrogen) and 100 ng of purified *M. bovis* DNA. The amplicons were initially cloned in the *pGEM-T Easy* (Promega) plasmid, following the manufacture's instructions. The cloned genes were sequenced in both directions using the BigDye Terminator v3.1 kit (Applied Biosystems) and analyzed for similarity in Blast program. After digestion with *Eco*RI, *mpb70* and *p27* were subcloned in *pGEX-3X* (GE), using the *Escherichia coli* DH5α strain as host cells. Recombinant proteins were purified through electroelution (Sá-Pereira et al., 2000). After induction of the gene expression with IPTG, the *E. coli* suspensions were cut vertically along the gel lysed by sonication and treated with 2x SDS-PAGE sample buffer. The lysates were run in preparative SDS-PAGE and subsequently immerged in 1M cold $C_2H_3KO_2$ (Neophytou et al., 1996). The major protein band from each lysate was cut vertically along the gel, and introduced into dialysis tubing (Sigma D9777-100FT) about 15 cm long, previously filled with 50 mM phosphate buffer, pH 6.0. The proteins were eluted from the gel slices at 100 V, for 3 h, at 4ºC. The eluted proteins were used for ELISA.

Serum samples

Sera from 90 heads of cattle from different herds from the state of Mato Grosso do Sul, Brazil, having tested either positive (53 animals) or negative (37 animals) on the comparative intradermal tuberculin test (CITT) following the

protocol of the Brazilian Agriculture Ministry (Brasil, 2004) were evaluated through ELISA. These samples were obtained by convenience, as the aim of the study was not for epidemiological analyses. Positive cases were notified by the veterinarian responsible for the skin test.

Enzyme-linked immunosorbent assays (ELISAs)

Polystyrene 96-well plates (Costar, 3590) were adsorbed with 0.5 µg/mL of antigen in phosphate buffer saline with 0.1% Tween 20 (PBST), pH 7.2, for 60 min at 37º C. The plates were then blocked with 100 µL/well of PBST with 5% skim milk for 60 min at 37º C. After five washes with PBST, 100 µL/well of control and test sera diluted 1:1000 in PBST were incubated for 60 min at 37º C. The plates were washed five times with PBST and 100 µL of monoclonal antibody anti-bovine IgG (heavy chain) horseradish peroxidase conjugate (Sigma, A5295), diluted 1:15,000 in PBST, were added to each well. The plates were incubated for 30 min at 37º C and after five washes, 50 µL/well of chromogen/substrate Fast OPD (Sigma, P9187) were added. The reactions were stopped with 3N HCl and the results were read on an EL-800 ELISA reader (Bio-Tek), with a 490-nm filter.

The ELISA cutoff was determined by Receiver Operating Characteristic (ROC) analysis (Zweig & Campbell, 1993), using the MEDCALC v.10.3.0.0 program. For the best cutoff selected, sensitivity and specificity were also determined for each test. Differences between tests were determined using the chi-square test (Microsoft®).

RESULTS AND DISCUSSION

In a Blastn search for homology, the cloned *mpb70* gene (Brazilian isolate of *M. bovis*) displayed 100% identity with homologues in the genomes of *M. bovis* Tokyo (AP10918), *M. tuberculosis* F11 (CP000717) and *M. tuberculosis* H37Ra (CP000611). The cloned *p27* gene displayed 99% identity with homologues in the genomes of *M. tuberculosis* H37Ra, *M. bovis* BCG Pasteur 1173P2 and *M. bovis* subsp. *bovis* AF2122/97. In Blastp, the deduced amino acid sequence of MPB70 from the present study displayed 100% identity with homologues of *M. tuberculosis* CDC1551 (NP_337454), *M. tuberculosis* H37Rv (NP_217391) and *M. bovis* AF2122/97 (NP_856545). For p27, the deduced amino acid sequence displayed 99% identity with homologues in *M. tuberculosis* CDC1551, *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv.

The high degree of conservation of *mpb70* and *p27* in the Brazilian isolate of *M. bovis* detected both at the nucleotide and amino acid levels when compared to other isolates is an important characteristic for diagnostic antigens. This demonstrates that major epitopes are shared among different isolates of the microorganism and directly reflects the sensitivity of the diagnostic test.

For ELISA-MPB70, a cutoff point > 0.354 was selected and sensitivity and specificity were 88.7% and 94.6%, respectively (Fig. 1). Therefore, for this cutoff point, there were six (11.7%) false-negative reactions among the 53 serum samples from cattle tested positive on the CITT as well as two (5.4%) false-positive reactions among 37 serum samples from cattle tested negative on the CITT. For ELISA-p27, sensitivity was 98.1% and specificity was 91.9% using a cutoff point of > 0.377. Thus, there was only one false-negative reaction (1.9%) among the 53 samples tested positive on the CITT and only three (8.1%) false-positive reactions among the 37 samples tested negative on the CITT (Fig 2). The ELISA absorbances were displayed in the figure 3.

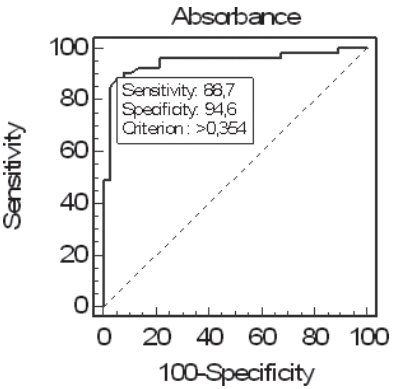

Figure 1. ROC Curve of ELISA with recombinant MPB70 and sera from 53 cattle tested positive and 37 cattle tested negative on the comparative intradermal tuberculin test.

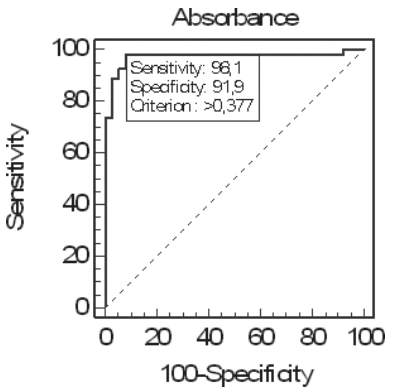

Figure 2. ROC Curve of ELISA with recombinant p27 and sera from 53 cattle tested positive and 37 cattle tested negative on the comparative intradermal tuberculin test.

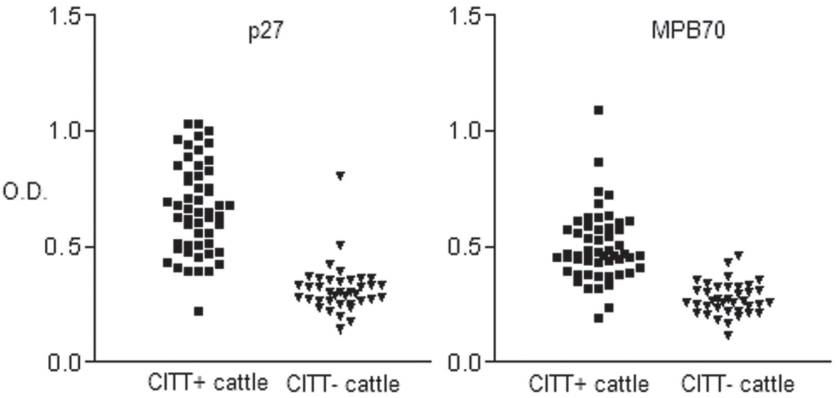

Figure 3. Distributions of absorbances of ELISA with recombinant MPB70 or p27 of *Mycobacterium bovis*.

With regard to specificity, the tests for recombinant p27 and MPB70 were statistically similar ($p > 0.05$). Nevertheless, the sensitivity of ELISA with p27 was significantly higher than that with MPB70 ($p = 0.05$), suggesting that further studies should be carried out on the function and antigenicity of p27.

Studies on the diagnosis of bovine tuberculosis are currently focused on the standardization of assays using recombinant proteins (Lightbody et al., 1998; Lightbody et al., 2000). Serological assays with the *M. tuberculosis* complex and recombinant antigens allow an increase in specificity and the fast screening of herds with infected cattle, which can be tested later individually by using the tuberculin skin test.

MPB70 is an immunodominant antigen of *M. bovis* (Fifis et al., 1991) and is a very stable, active component of *M. bovis* tuberculin (Harboe et al., 1990). It is able to elicit delayed hypersensitivity responses, the proliferation of T lymphocytes and the production of antibodies in cattle infected with *M. bovis* (Harboe et al., 1990; Fifis et al., 1991).

The CITT was used as the *ante-mortem* reference test for the classification of cattle as either positive or negative for tuberculosis in this study. It was not possible to follow the slaughter of the animals. The false-positive reactions found in the present study may have been caused by chronically-infected anergic cattle (Harboe et al., 1990; Surujballi et al., 2002; Aagaard et al., 2006), which may exhibit antibodies against *M. bovis* albeit in the absence of a cell response. If this hypothesis is correct, the combination of cellular and antibody tests would improve the diagnostic coverage of bovine tuberculosis, as anergic cattle could be detected by ELISA. The false-negative reactions found in the present study may be attributed to the common late onset humoral responses in the course of infection with *M. bovis* (Ritacco et al., 1991). Therefore, serological methods may exhibit a lower

sensitivity than cellular methods regarding the diagnosis of bovine tuberculosis in the early stages of infection. Another possibility is the false-positive status of some animals in the skin test, due to cross-reactions with environmental mycobacteria. This last hypothesis is less probable, as we have used the CITT to define positive or negative cattle.

In conclusion, recombinant MPB70 and recombinant p27 are promising for use in enzyme-linked immunosorbent assays for the detection of antibodies against *M. bovis,* which, when associated to the tuberculin skin test, could improve the diagnostic coverage of bovine tuberculosis. Future studies with definitive follow-up of skin test positive cattle would provide more accurate data of the potential of these antigens for serological diagnosis of bovine tuberculosis.

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