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Molecular Differentiation of Rural Tuberculosis Cases in Mato Grosso: Investigating the *Rv2807* and *TbD1* Genes by Nested q-PCR

Diferenciação Molecular de Casos de Tuberculose Rural em Mato Grosso: Investigando os Genes Rv2807 e TbD1 por Nested q-PCR

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Abstract

Among infectious diseases, human tuberculosis (TB) remains one of the major public health problems. Information regarding groups vulnerable to this disease caused by *Mycobacterium bovis* is scarce both nationally and internationally. Therefore, this research aimed to investigate the occurrence of *M. bovis* among patients with sputum positive for the *Mycobacterium tuberculosis* complex (TBMC), from rural areas of some cities in Mato Grosso. To this end, the target gene *Rv2807* was

evaluated using Nested q-PCR to confirm TBMC, and for *M. bovis* by detecting the *TbD1* region, directly from DNA extracted from nine sputum samples with positive TBMC cultures provided by LACEN-MT, selected from 61 individuals diagnosed with pulmonary tuberculosis by LACEN/MT between 2017 and 2023. After heat inactivation using the Dneasy Blood & Tissue kit - Qiagen, DNA was extracted according to the manufacturer's instructions. The nine samples tested were confirmed as belonging to the *Mycobacterium tuberculosis* Complex through the presence of the *Rv2807* gene. However, the *TbD1* region was not observed, revealing the absence of *Mycobacterium bovis* among patients positive for the *Mycobacterium tuberculosis* Complex. Even so, this highlights the importance of investing in molecular testing for species belonging to the *Mycobacterium* genus in samples positive for both bovine and human tuberculosis. It should be emphasized that tuberculosis remains a serious cause of infectious health problems in Brazil, making it necessary to investigate the species detected in confirmed TB cases more precisely, especially in rural populations and agricultural workers.

Keywords: *Mycobacterium bovis*. Tuberculosis. *Mycobacterium tuberculosis* Complex. One Health.

Resumo

Entre as doenças infecciosas a tuberculose humana (TB) permanece sendo um dos grandes problemas de saúde pública. E informações sobre grupos vulneráveis à esta patologia desencadeada pelo *Mycobaterium bovis* são poucas nacional e/ou internacionalmente. Portanto, esta pesquisa objetivou averiguar a ocorrência *M. bovis* entre pacientes com escarros positivos para o complexo *Mycobacterium tuberculosis* (CMTB), provenientes da zona rural de algumas cidades de Mato Grosso. Para tanto, se avaliou através do Nested q-PCR para confirmação do CMTB o gene alvo *Rv2807*, e para *M. bovis* pela detecção da região *TbD1*, diretamente do DNA extraído de nove amostras de escarro com cultura positiva para CMTB cedidos pelo LACEN-MT, selecionados entre 61 indivíduos diagnosticados com Tuberculose pulmonar pelo LACEN/MT no período de 2017 a 2023. Após a inativação pelo calor, e utilizando o kit *Dneasy Blood & Tissue*- Qiagen, extraiu-se o DNA. As nove amostras testadas foram confirmadas como pertencentes ao Complexo *Mycobacterium tuberculosis*, através da presença do gene *Rv2807*. No entanto, não se observou a presença da região *TbD1*, revelando a ausência de *Mycobacterim bovis* entre os pacientes positivos para o Complexo *Mycobacterium tuberculosis*. Mesmo assim se destaca aqui a importância no investimento de testagem molecular para espécies pertencentes ao gênero *Mycobacterium* diante de amostras positivas para tuberculose tanto bovina, quanto humana. Pode se ressaltar que a tuberculose continua uma causa grave de agravo a saúde de origem infecciosa no Brasil, sendo necessário a investigação mais precisa das espécies detectadas nos casos de TB confirmados, principalmente em população rural e trabalhadores rurais.

Palavras-chave: Complexo *Mycobacterium tuberculosis. Mycobacterium bovis.* Tuberculose. Saúde Única.

1 Introduction

Human tuberculosis is caused by mycobacteria belonging to the *Mycobacterium Tuberculosis* Complex (MTBC). Officially, the MTBC includes the following species: *M. tuberculosis*, *M. bovis*, *M. bovis* BCG; *M. africanum*, *M. microti*, *M. caprae*, *M. pinnipedii*, and *M. canetti* (Brasil, 2022; Lin *et al*., 2014). There are 20 species of mycobacteria that can potentially cause tuberculosis and another 39 species of this genus that rarely cause this pathology (Brasil, 2022). However, the eight species

mentioned above have already been detected as causative agents of tuberculosis, with *M. tuberculosis* responsible for 98% of cases and *M. bovis* implicated as an agent in 1% or 2% of human tuberculosis cases (Brasil, 2022; Lin *et al*., 2014).

Tuberculosis (TB), considered a zoonosis, is caused by *Mycobacterium bovis*, a bacterium belonging to the *Mycobacterium tuberculosis* complex (MTC). While it can cause tuberculosis in cattle and buffalo (BTB), it can also trigger the disease in a variety of mammals, including humans (Anjos *et al*., 2022a; Carneiro *et al*., 2020; Carvalho *et al*., 2016). This infection is clinically and pathologically indistinguishable from that caused by *M. tuberculosis*, and is caused by the consumption of contaminated animal products such as unpasteurized milk and dairy products (Anjos *et al*., 2022a; Carneiro *et al*., 2020; Carvalho *et al*., 2016).

Tuberculosis in humans is diagnosed through bacterioscopy of specimens stained using the Ziehl-Neelsen method for Acid-Fast Bacilli (AFB) (Brazil, 2022). Diagnosis can also be made by culturing in egg-based media (Lowenstein-Jensen or Ogawa Kudoh agar, both with sodium para-nitrobenzoate - PNB), or with Middlebok 7HA liquid and solid media (Brazil, 2022). Differentiation involves macroscopic and microscopic analysis of colonies, immunochromatography, niacin, and PNB tests (diagnosis of CMTB) (Brazil, 2022). Identification of *Mycobacterium* species is performed using PNB (nitrobenzoic acid), thiophene-2-carboxylic acid (TCH), streptomycin (SM), nitrate reduction, and niacin tests (Brazil, 2022). There is also molecular diagnosis, which is performed using the rapid molecular test for tuberculosis (TRM-TB). The Xpert® MTB/RIF Ultra test was developed using the same GeneXpert® platform, and it utilizes two different multicopy amplification targets (IS6110 and IS1081 genes) (Brazil, 2022).

According to Carneiro *et al*. (2020), "Molecular techniques are increasingly used to support conventional methods, both for the identification and confirmation of *M. bovis* strains, and for molecular epidemiology." The identification and characterization of *M. tuberculosis* and *M. bovis* can be performed by investigating their genomic variation (Amadio et al., 2005; Araújo *et al*., 2014). The *TdD1* region of the *mmpS6* gene is present in *M. bovis* and absent in the *mmpS6* of modern *M. tuberculosis* strains (Anjos *et al*., 2022a,b; Araújo *et al*., 2014). The *Rv2807* gene is specific for the *Mycobacterium tuberculosis* Complex (MTBC); its use in conjunction with *TbD1* should be considered for the detection of *M. bovis* (Anjos *et al*., 2022a,b; Araújo *et al*., 2014).

There is insufficient data to support control actions for zoonotic tuberculosis (*Mycobacterium bovis*) in low- and middle-income countries. This is particularly true regarding information on risk factors, vulnerable groups, and disease burden (Couto *et al*., 2022; Villa *et al*., 2023). A One Health

approach that includes control strategies such as joint actions between human and animal health programs, with integrated surveillance, is vitally important for controlling and producing current epidemiological information on TB (Couto *et al*., 2022; Villa *et al*., 2023).

In this context, this study aimed to investigate the occurrence of *M. bovis*, through molecular analysis (Nested q-PCR) using the *Rv2807* and *TbD1* genes, among strains previously classified as belonging to CMTB, isolated from sputum of individuals from rural populations in Mato Grosso, with symptoms and diagnosed with tuberculosis using the diagnostic method recommended by the Ministry of Health.

2 Material and Methods

The samples evaluated here were selected from 61 individuals diagnosed with pulmonary tuberculosis by LACEN/MT (Central Laboratory of the state of Mato Grosso) between 2017 and 2023. The inclusion criterion was that the individual resided or worked in a rural area and had biological material stored in the aforementioned laboratory. Therefore, in this study, nine strains previously identified using the culture method recommended by the Ministry of Health (Brazil, 2022) were evaluated in duplicate. These strains were provided by LACEN/MT and, in the Molecular Biology Laboratory of the University of Cuiabá, were designated A through I (A1 and A2) and so on.

The strains, upon arrival at the Molecular Biology laboratory of the University of Cuiabá-UNIC, were inactivated at 80 ºC for 20 minutes (Doig *et al*., 2002). After inactivation of the isolates, the samples were subjected to DNA extraction using the Dneasy Blood & Tissue Kit - QIAGEN, and the DNA obtained was quantified using Biodrop.

The DNA of the strains after extraction was analyzed by Nested q-PCR for detection of the *Mycobacterium tuberculosis* Complex (MTBC) through the target gene *Rv2807* and *M. bovis* through detection of the *TbD1* region, according to Araújo *et al*. (2014), modified by Carvalho *et al*. (2015). Nested q-PCR analyses were performed using the StepOne Real Time instrument (Applied Biosystems™), and those samples with a peak above the target limit were considered positive, and those below negative.

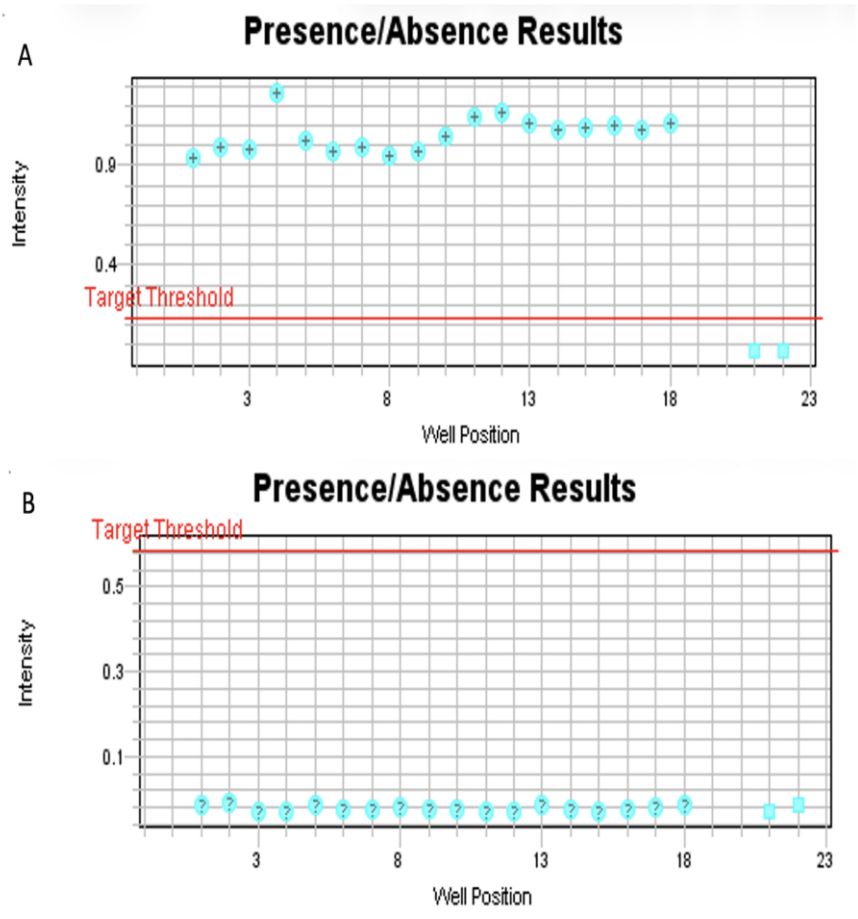
3 Results and Discussion

The nine strains were isolated from sputum collected from male patients with a maximum education level equivalent to elementary school. The material was decontaminated using the Petroff method and identified by automated liquid culture in Ogawa medium. The strains were selected from

61 individuals diagnosed with pulmonary tuberculosis by LACEN/MT between 2017 and 2023. The genes of the nine samples diagnosed by the methods recommended by the Ministry of Health were then subjected to nested qPCR using the *Rv2807* primers and the *TbD1* gene.

This confirmed that they belonged to the *Mycobacterium tuberculosis* Complex (MTBC), showing amplification of the *Rv2807* gene (Figure 2, graph A). However, none of them showed amplification for the *TbD1* island, and were therefore negative for the *Mycobacterium bovis* species (Table 1 and Figure 1).

Figure 2 – Graph (A) represents the reactions of the analysis in Real Time StepOne (Applied Biosystems™), of the *Rv2807* gene, and graph (B) to *TbD1*, representing the presence and/or absence of these genes. In these two graphs (A and B), the samples are distributed in duplicate, making eighteen points, with points 1 and 10 relating to sample A, and so on, ending at points 9 and 18 relating to sample I. Only those points above the target threshold were considered positive (gene present)



Source: research data.

Table 1 - Microbiological culture and Nested q-PCR of sputum samples isolated from individuals from rural populations in Mato Grosso, with symptomatology

Samples	Results		
	Culture Method	Nested q-PCR	
		Gene Rv2807 for CMTB	TbD1 gene for *M. bovis*
1	+	+	-
2	+	+	-
3	+	+	-
4	+	+	-
5	+	+	-
6	+	+	-
7	+	+	-
8	+	+	-
9	+	+	-
Total	**9 (9/9)**	**9 (9/9)**	**0 (0/9)**
%	**100%**	**100%**	**0%**

Source: research data.

This result is consistent with what is described in the literature, since in humans the disease caused by *M. bovis* is anatomically different, occurring as an extrapulmonary disease (Rocha *et al*., 2011). The use of a gene targeting the Myco*bacterium tuberculosis* complex (*Rv2807*) and the *M. bovis* species (*TbD1*) is important in this differential diagnosis. This is because the *TbD1* region of the *mmpS6* gene is present in *M. bovis* and absent in the *mmpS6* of modern strains of *M. tuberculosis* (Anjos *et al*., 2022a,b; Araújo *et al*., 2014). The *Rv2807* gene is specific for the *Mycobacterium tuberculosis* Complex (MTBC), and its use in conjunction with *TbD1* should be considered for the detection of *M. bovis* (Anjos *et al*., 2022a,b; Araújo *et al*., 2014).

The occurrence of *M. bovis* in cattle herds has been described in the state of Mato Grosso, in dairy cattle (Néspoli *et al.*, 2016; Carvalho *et al*., 2015), as well as in beef cattle (Furlaneto *et al*., 2012; Rocha *et al*., 2020; Barcelos *et al*., 2021; Anjos *et al*., 2022a). In rural populations in Brazil, there is a habit of consuming raw milk and producing cheese with raw milk, which is concerning because there are reports of *M. bovis* isolated from milk and raw cheese in Brazil (Carneiro *et al*., 2017; Carvalho *et al*., 2015; Cezar *et al*., 2016; Néspoli *et al*., 2016). Given that the *Rv2807* and *TbD1* genes were used for the detection of the *Mycobacterium tuberculosis* Complex and *M. bovis*, respectively, by Barcelos *et al*. (2021) and Anjos *et al*. (2022a) in beef cattle from Mato Grosso, and only the *TbD1* region by Araújo *et al*. (2014), evaluating samples from cattle and buffalo from commercial slaughterhouses in Brazil. Therefore, amplifications of these genes in beef cattle justify the importance of investigating them in sputum from humans positive for Tuberculosis.

In order to control and eradicate tuberculosis caused by *M. bovis*, it is important to implement integrated surveillance, with the aim of improving human, animal, and ecosystem health through

effective interventions, from a One Health perspective (Silva *et al*., 2025; Couto *et al*., 2022; Villa *et al*., 2023).

4 Conclusion

The investigation of sputum samples, carried out through molecular analysis (Nested q-PCR) using the *Rv2807* and *TbD1* genes, confirmed the identification of the isolates as belonging to the *Mycobacterium tuberculosis* Complex (TBMC). However, no strains of *Mycobacterium bovis* were detected among the isolates originating from sputum of individuals from rural populations in Mato Grosso, with symptoms and diagnosed with tuberculosis through the conventional culture method.

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Authors' Contributions:
Substantially contributing to the conception or design of the study: Lauren Cristiane Leite Ocampos and Ricardo César Tavares Carvalho; *acquiring, analyzing, or interpreting data for the work*: Lauren Cristiane Leite Ocampos, Andressa Ricci Biz Messias, Doralcide Terumi Takahar, Elaine Cristina Oliveira, Klaucia Rodrigues Vasconcelos, Tony Edgley Catão Tenório, Adelino Cunha Neto, and Ricardo César Tavares Carvalho; *critically elaborating or revising the article regarding its intellectual content*: Lauren Cristiane Leite Ocampos, Andressa Ricci Biz Messias, Doralcide Terumi Takahar, Elaine Cristina Oliveira, Klaucia Rodrigues Vasconcelos, Tony Edgley Catão Tenório, Adelino Cunha Neto, and Ricardo César Tavares Carvalho; *approving the final version of the article*: Adelino Cunha Neto and Ricardo César Tavares Carvalho; *agreeing to and being responsible for all aspects of the work*: Lauren Cristiane Leite Ocampos, Andressa Ricci Biz Messias, Adelino Cunha Neto, and Ricardo César Tavares Carvalho.

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