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Bovine tuberculosis diagnosis in the state of Bahia, Brazil, using the multiplex PCR technique

Diagnóstico da tuberculose bovina no estado da Bahia, Brasil, utilizando a técnica de PCR multiplex

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Abstract: The rapid diagnosis of bovine tuberculosis (BT) allows official surveillance institutions to act in areas that could be potential hotspots for the spread of the disease. The study aimed to identify the Mycobacterium bovis agent by multiplex PCR of isolated colonies. Through the official inspection service (Federal/State), samples suggestive of BT were collected during the post-mortem inspection of bovine carcasses in slaughterhouses and sent to the laboratory for complementary diagnostics. The samples were analyzed using bacteriological culture and Ziehl-Neelsen staining. In isolates characterized as Acid-Alcohol Resistant Bacilli (BAAR), the multiplex PCR technique confirmed the agent Mycobacterium bovis. Nine hundred ninety-one thousand three hundred sixteen cattle carcasses were inspected, collecting 32 BT-suggestive samples in from animalsfrom 30 municipalities. Of these, 28.1% (9/32) showed growth on bacteriological culture media with BAAR. The BAAR isolates submitted to multiplex PCR confirmed the presence of M. bovis. The multiplex PCR technique associated with bacteriological examination and applied to post-mortem findings enabled the diagnosis of M. bovis in the municipalities sampled in the Bahia state.

Keywords: Inspection; Mycobacterium bovis; multiplex PCR; epidemiological monitoring; public health.

Resumo: O diagnóstico rápido da tuberculose bovina (TB), permite a atuação das instituições oficiais de vigilância em áreas que possam vir a constituir possíveis focos de disseminação da doença. Objetivou-se com essa pesquisa identificar o agente Mycobacterium bovis através da PCR multiplex das colônias isoladas. Através do serviço de inspeção oficial (Federal/ Estadual), coletou-se amostras sugestivas de TB durante a inspeção post-mortem de carcaças bovinas em matadouros-frigoríficos e as encaminhou ao laboratório para os diagnósticos complementares. As amostras foram analisadas por meio de cultura bacteriológica e coloração de Ziehl-Neelsen. Nos isolados caracterizados por Bacilos Ácido-Álcool Resistente (BAAR), aplicou-se a técnica de PCR multiplex visando a confirmação do agente Mycobacterium bovis. Foram inspecionadas 991.316 carcaças de bovinos, onde 32 amostras sugestivas de TB foram coletadas, provenientes de animais de 30 municípios. Destas, 28,1% (9/32) apresentaram crescimento em meios de cultura bacteriológicos com BAAR. Os BAAR isolados

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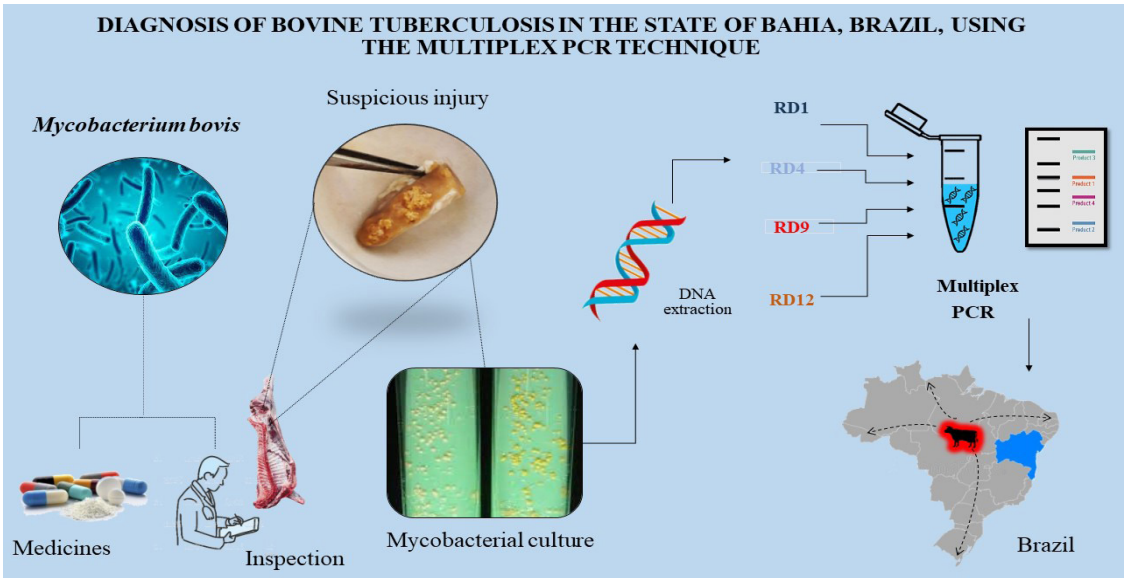
submetidos à PCR multiplex confirmaram a presença de M. bovis. A técnica de PCR multiplex associada ao exame bacteriológico e aplicada aos achados post-mortem permitiu o diagnóstico de M. bovis nos municípios amostrados do estado da Bahia.

Palavras-chave: Inspeção; Mycobacterium bovis; PCR multiplex; vigilância epidemiológica; saúde pública.

Graphical abstract:



1. Introduction

The World Health Organization classifies bovine tuberculosis (BT) as a zoonosis, representing a global public health concern. The disease mainly affects cattle, which have high morbidity and mortality rates, other domestic animals, and humans, mainly in developing countries. In these countries, cases of human infection can reach 20% [(1)]. BT is considered a neglected disease, in which the lack of information favors its advancement and spread.

BT is a chronic bacterial infectious disease caused by *Mycobacterium bovis*, belonging to the *Mycobacterium tuberculosis* Complex (MTBC), responsible for most human and animal tuberculosis cases [(2)]. The development of nodular granulomatous lesions characterizes this disease and can affect any tissue or organ; however, it primarily affects the respiratory tract, lymph nodes, mainly retropharyngeal, bronchial, and mediastinal and, occasionally, peritoneum, pleura, spleen, liver, and intestines [(3)].

BT is not only a health problem but also an economic concern. Cattle and buffaloes are the most affected species by the disease, causing considerable financial losses of up to 20% derived from the reduction of milk and meat production, carcass condemnations, reproductive alterations, and international market restrictions [(4)]. The Ministry of Agriculture, Livestock and Supply of Brazil (MAPA) established in 2001 the "National Program for the Control and Eradication of Brucellosis and Tuberculosis" – NPCEBT, to reduce the disease prevalence and incidence [(3, 5)].

Mycobacterial isolation is the "gold standard" test for diagnosing tuberculosis [6]. However, it is impossible to identify the agent species, requiring complementary molecular methods [7], such as the multiplex polymerase chain reaction (PCR) technique. The multiplex PCR technique uses specific genomic regions to identify members of the *Mycobacterium tuberculosis* Complex, complementing bacteriological cultivation by identifying the agent and replacing biochemical tests that otherwise require more time and technical knowledge. According to Zanini et al.[8], the PCR technique effectively detects members of the *M. tuberculosis* complex, providing a rapid and valuable diagnosis for *M. bovis* in bovine tissue samples suggestive of BT.

The more precise disease detection through the multiplex PCR molecular technique will enable epidemiological surveillance in its focal points and help in the disease control by the National Program for the Control and Eradication of Brucellosis and Tuberculosis – NPCEBT in Bahia. Furthermore, it will reduce the spread of the disease in the herd, economic losses caused by carcass condemnation, and the risk of the human population contracting zoonotic tuberculosis [9]. The present project investigated the presence of *M. bovis* using bacteriological isolation followed by multiplex PCR to differentiate the members of the *Mycobacterium tuberculosis* Complex from the genomic regions of differentiation.

2. Materials and methods

2.1 Collecting and sending samples

The study was performed in the state of Bahia, which ranks as the 5th most extensive state in Brazil, with a 567,295 km² surface, comparable to the territorial extension of France. BT-suggestive samples from any animal tissue or organ displaying suspicious nodular lesions were collected during the cattle *post-mortem* examination at the official inspection between March 2020 and December 2021. The samples were sent to the Laboratory of Mycobacteriology at UESC in Falcon-type tubes (50mL) containing a saturated solution of sodium tetraborate ($Na_2[B_4O_5(OH)_4]\cdot 8H_2O$, 140g/L) as a preservative and, together with the samples, animal identification forms were forwarded containing: municipality of origin, owner, date of sample collection, breed, age, sex, description of the tissue/organ sent and responsible official inspector. The Ethics Committee for the Use of Animals (CEUA) of the State University of Santa Cruz (Brazil) approved the study under registration No. 017/22, and the Bahia Livestock Defense Agency (ADAB) gave its consent to publish the results.

2.2 Processing of lesions and bacteriological isolation

The suggestive samples were manually macerated into 1g (one gram) of tissue and then decontaminated with 1-Hexadecylpyridinium chloride (HPC) at 1.5% for 30 minutes. They were then centrifuged to remove the HPC, resuspended in 0.84% saline solution for inoculation into Stonebrink-Lesslie and Lowenstein-Jensen culture media, and incubated at 37°C for up to 90 days [10]. The colonies observed in the culture medium were fixed by smears and stained using the Ziehl-Neelsen method. A bacterial suspension of the acid-alcohol resistant bacilli (BAAR)

isolates was made in 200µL of TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8.0). Thermal lysis was carried out To obtain the DNA by incubation at 100°C for 30 minutes. The DNA from the samples was quantified on a NanoDrop 2000 (Thermo Scientific®, USA).

2.3 Multiplex polymerase chain reaction (PCR)

The multiplex PCR technique was carried out using pairs of primers based on the different genomic regions of the *Mycobacterium tuberculosis* complex: RD1, RD4, RD9, and RD12 [(11)], with RD1(present) for the identification of *M. bovis*; RD4 (present) for *M. africanum, M. caprae, M. pinnipedii,* and *M. microti*; RD9 (present) for *M. tuberculosis* and RD12 (absent) for *M. canettii, M. caprae, and M. bovis*. Of each sample, 4µL were used, containing 50ng of DNA, 0.2 Units of Platinum Taq DNA polymerase enzyme (INVITROGEN), 5.0 µL PCR buffer, 2.0 mM MgCl2, 0.4 mM of each dNTP, 0.5 µM of each primer. DNA from *M. bovis* and *M. tuberculosis* already belonging to the Laboratory of Mycobacteriology at UESC represented the positive control, and ultrapure water (LABTRADE®) was the negative control. The reactions were performed in a thermocycler (Bio-Rad iCycler® iQ5) under the following conditions: initial incubation at 95°C/5 min, followed by 45 94°C/min, 62°C/min cycles and 72°C/min and a final extension at 72ºC for 10 minutes. The electrophoresis (60V/cm) separated the amplification products on a 3% agarose gel in 1X TBE buffer and visualized by staining with SYBR Safe (INVITROGEN®).

2.4 Statistical analysis

The Fisher's exact test assessed the relationship between sex and the positivity index, considering a 5% significance level. The hypotheses are:

H0: Sex = index of positive animals
H1: Sex ≠ index of positive animals

3. Results

From March 2020 to December 2021, the official inspection system inspected 991,316 carcasses in 16 slaughterhouses in 30 municipalities of the state of Bahia. Among all slaughtered animals, 32 displayed tuberculosis-suggestive lesions (4×10^{-4}), 18 females and 14 males older than 36 months.Fisher's test displayed no significant relationship between sex and positivity index (p = 0.1317) (Table 1).

Table 1. Contingency table displaying the relationship between gender and positivity rate

Sex	Observed positivity			Expected positivity		
	Positive	Negative	Total	Positive	Negative	Total
Male	6	8	14	3,94	10,6	24
Female	3	15	18	5,06	12,94	18
Total	9	23	32	9	23	32

BT-suspected lesions had a granulomatous, nodular, caseous, or calcified aspect. The lymph nodes corresponded to most of the lesions, reaching 68.75% (22/32) of the samples sent, followed by lung with 12.5% (4/32), musculature with 9.37% (3/32), liver with 3.12% (1/32), tongue 3.12% (1/42), and abomasum with 3.12% (1/42). The bacteriological examination revealed that 34.37% (11/32) of the samples formed colonies in the specific culture media. The colonies showed a cream to yellowish color, rounded edges, rough surface, and dysgonic growth in the culture medium: 90.9% (10/11) samples grew only in the Stonebrink-Lesslie (ST) medium, and 9.1% (1/11) developed in both Stonebrink-Lesslie and Lowenstein-Jensen (LJ) media (Figure 1).

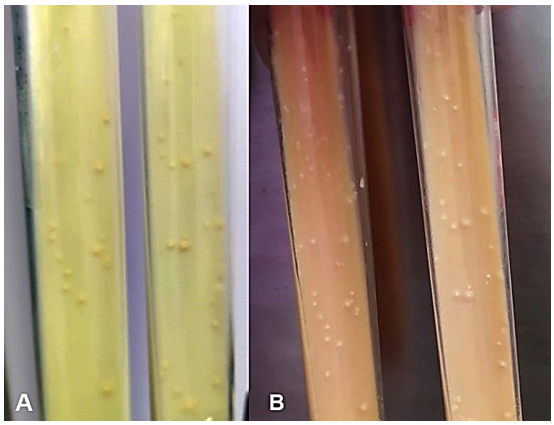


Figure 1. Positive bacteriological test showing the formation of colonies on Stonebrink-Leslie (A) and Lowenstein-Jensen (B)

The colonies' formation time varied between 14 and 84 days, averaging 39 days. Nine of the 11 positive samples in the culture media had BAAR, totaling 28.1% of the analyzed samples. The positive samples originated from the municipalities of Itagibá/BA, Tomar do Geru/SE (State of Sergipe), Ibicuí/BA, Gongoji/BA, Jaremoabo/BA, Euclides da Cunha/BA, Barreiras/BA, São Gonçalo dos Campos/BA, and Teodoro Sampaio /BA (Figure 2).

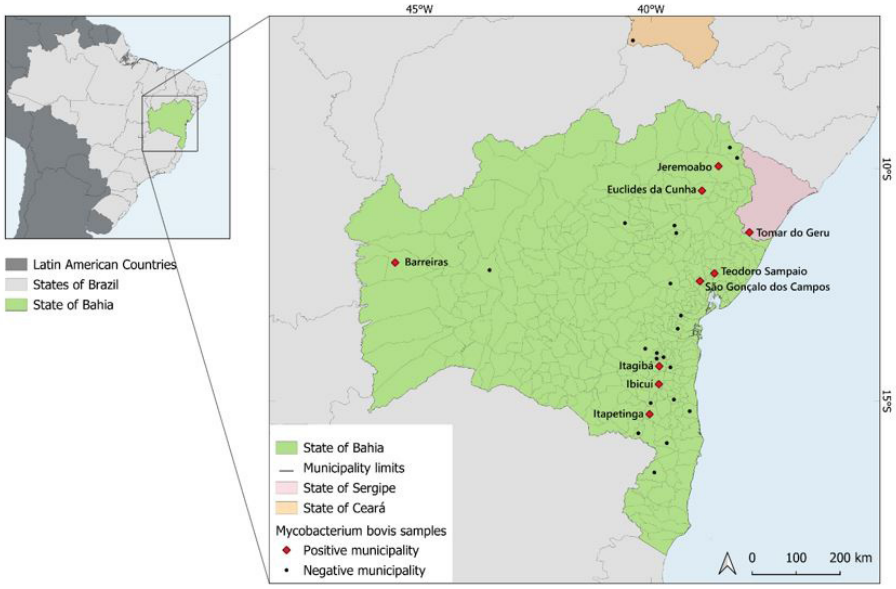


Figure 2. Map of Bahia with the municipalities sampled for the investigation of *Mycobacterium bovis* through bacterial culture and multiplex PCR. Black circles indicate the municipalities negative for the presence of the *Mycobacterium bovis* agent; red diamonds positive municipalities for *Mycobacterium bovis*.

All BAAR samples amplified the RD1, RD4, RD9, and RD12 regions through multiplex PCR (Figure 3), confirming the presence of *M. bovis.*

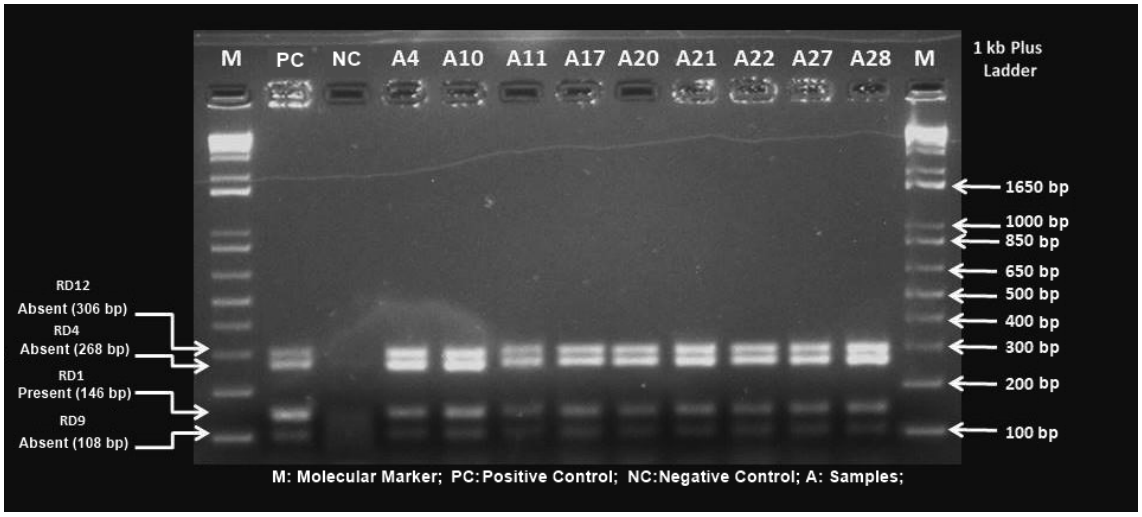


Figure 3. M- molecular marker; PC– positive control; NC - negative control; A4-A28 - animal samples. White arrows indicate the amplification of regions RD1 (146 bp), RD4 (268 bp), RD9 (108 bp), and RD12 (306 bp), confirming the presence of *M. bovis* in all samples.

4. Discussion

Suggestive tuberculosis samples came from 32 animals and 16 slaughterhouses, where the official inspection system inspected 991,316 beef carcasses. The bacteriological culture, staining, and multiplex PCR techniques confirmed 9/32 (28.1%) of the suspected BT samples collected. These nine samples came from nine animals aged over 36 months from nine municipalities, eight municipalities in Bahia/BA and one in Sergipe/SE, on the border with Bahia. BT was investigated in Bahia in a study carried out between 2009 and 2012, showing 56% (14/25) positivity in samples suggestive of BT collected from slaughterhouses and confirmed by the multiplex PCR technique [10]. The quoted study described the primary lesions' location: lymph nodes, lungs, carcass, liver, tongue, and abomasum. The lungs, pulmonary lymphatic chains, lymph nodes, and liver [12] typically host the gross tuberculosis lesions. Other authors also report a predominance in the involvement of mediastinal lymph nodes, followed by lung and liver [10,13,14].

Regarding growth in specific culture media, the bacteria required between 14 and 94 days to form colonies, with an average of 39 days. These findings resemble those described by other authors [12,15,16], who found mean growth times of 21 days, 36 days, and 28 days, respectively. Although all samples in the present study grew on the ST medium, only one grew on both ST and LJ media. The sample that developed in the LJ medium showed smaller colonies than in the ST. The reduced growth may have depended on the absence of pyruvate in the LJ medium, using glycerin as a substrate source. This difference favors the development of other mycobacteria than *M. bovis* [17]. However, *M. bovis* adapted to the culture medium, growing even without pyruvate [18].

Statistical analysis revealed that the positivity rate does not correlate with the animals' sex. Numerous other factors may affect the number of positive cases, including the animal's immunity, which can isolate or spread the infection through nasal, salivary, vaginal, uterine, urinary, sperm, and feces secretions [19], as tuberculosis is an infectious disease. In addition, the older the animal, the greater the probability of having had contact with other infected animals due to the animal segregation in the property according to their development [20].

This work observed that all BT-positive animals, regardless of sex, were older than 36 months. This fact reinforces the extended period that these animals may remain on the property until the slaughter, thus contributing to the spread of the disease in the herd [21]. Besides, cows affected by the tuberculosis bacillus have a good appearance, and their owners sell them instead of discharging them. This behavior explains why it is possible to find sick cows from legal breeders, even if dairy farm veterinarians recommend their slaughter. These animals become dissemination sources, causing risks to handlers since suspected BT lesions can be isolated and observed only at slaughter based on *post-mortem* findings[2]. Another factor that corroborates the spread of TB is the lack of tuberculin testing (TT) on small farms. The program's guidelines preconize the discharge of TT-positive animals. The small producers consider it an economic and production loss, as they are still full-producing, do not search for qualified veterinarians or the state defense agency responsive to perform the tests, and sell the animals illegally.

The bacteriological examination associated with Ziehl–Neelsen staining and multiplex PCR eliminates false positive results. Even if colonies form, this does not indicate that they are strains of *M. bovis*, as there may be the growth of other microorganisms, such as gram-positive bacteria that, when stained, will not be visualized as BAAR. In the same form, some BAAR-positive bacteria are not *M. bovis*. In the present work, only one sample was negative for staining, not indicating the isolate as BAAR. Santos[7] already observed a similar result, as the author applied the technique of bacteriological cultivation followed by Ziehl–Neelsen staining in suggestive BT samples collected at open-air markets. Samples that showed colony growth in the culture media were negative for the Ziehl–Neelsen staining technique.

In the present research, multiplex PCR enabled a more precise identification of the etiological agent based on the amplification of genomic regions of MTBC difference. The specific genome regions allow us to differentiate them by presence or absence, showing bands in the agarose gel with different base pair (bp) sizes. The amplified regions on the gel were RD1 (146 bp), RD4 (268 bp), RD9 (108 bp), and RD12 (306 bp) (Figure 3), confirming the presence of the *Mycobacterium bovis* agent in all BAAR-positive isolates. Other authors [10,22] also used multiplex PCR associated with bacterial culture to identify members of the MTBC complex to provide a more accurate etiological diagnosis and describe similar results.

Despite the significant advances in tuberculosis diagnosis, studies have not yet identified an ideal test for routine tuberculosis diagnosis in cattle, and it is essential to increase investment in research to solve this critical issue in the fight against the disease [23]. Furthermore, Cosivi [15] points out the impossibility of using any diagnostic method alone and that complementary

techniques (TT, bacteriology, staining, PCR techniques) should always be used to obtain an effective and complete diagnosis.

The slaughterhouses inspection service is crucial to track areas that may become outbreaks of bovine tuberculosis. Collecting samples suspected of BT and their cities of origin can provide epidemiological records, helping the activities of the NPCEBT. In this way, it will be possible to implement a control and monitoring system given the socioeconomic conditions of the municipalities in the Bahia state.

5. Conclusion

Bovine tuberculosis is present in cattle in the Bahia state, and applying the multiplex PCR technique associated with *post-mortem* examinations and bacteriology allowed the diagnosis and identification of *Mycobacterium bovis* with more precision in the highlighted municipalities in the state.

Declaration of conflict of interest

We affirm that there is no conflict of interest related to this article's writing, authorship, or publication.

Author contributions

Conceptualization: B. S. Ribeiro, A. V. Silva, B. M. Maciel and F. F. Alzamora. *Formal analysis:* H. F. Ferraz. *Methodology*: B. S. Ribeiro, A. V. Silva, B. M. Maciel and H. F. Ferraz. *Research:* B. S. Ribeiro. *Project administration*: F. F. Alzamora and B. M. Maciel. *Supervision:* F. F. Alzamora and B. M. Maciel. *Writing (review and editing):* B. S. Ribeiro and F. F. Alzamora.

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