http://dx.doi.org/10.1590/1678-4162-12394

Arq. Bras. Med. Vet. Zootec., v.74, n.2, p.338-344, 2022

Isolation and molecular identification of *Mycobacterium bovis* in tissue lesions of cattle slaughtered in slaughterhouses located in the State of Ceará

[*Isolamento e identificação molecular de* Mycobacterium bovis *em lesões teciduais de bovinos abatidos em matadouros-frigoríficos localizados no estado do Ceará*]

J.Q. *Amorim*[1], B.R. *Santos*[1], H.F. *Fehlberg*[1], A.V. *Silva*[1], F.F. *Ferreira*[2], J.N. *Costa*[3], F. *Alzamora Filho*[1]

[1]Universidade Estadual de Santa Cruz, *Campus* Soane Nazaré de Andrade, Ilhéus, BA, Brasil
[2]Agência de Defesa Agropecuária do Estado do Ceará, Fortaleza, CE, Brasil
[3]Universidade Federal do Recôncavo Baiano, Cruz das Almas, BA, Brasil

ABSTRACT

The objective of this research was to identify *Mycobacterium bovis* in lesions suggestive of tuberculosis in bovine carcasses in the State of Ceará, by means of bacteriological and molecular diagnostic tests. Between August 2017 and January 2019, the State inspection service (SIE) inspected 59,512 cattle, of which 7.4% (44 / 59,512) presented suggestive lesions. Of these animals, 68 samples were sent, of which 4.5% (31/68) located in the lung, 2.9% (20/68) in lymph nodes, 2.0% (14/68) in the liver, and 0.4% in the carcass (3/68). When performing bacteriological isolation, 15.9% (7/44) of bovines showed colony growth in the samples. The smears of the isolates were submitted to Zielh-Neelsen staining and all confirmed acid-fast bacilli. The polymerase chain reaction identified all isolates 100% (7/7) as *M. bovis.* The association of diagnostic techniques allowed to identify the presence of the agent in the State and the molecular analysis proved to be a beneficial technique in the monitoring of bovine tuberculosis and can be used as an auxiliary method in the bovine tuberculosis control and eradication program in the State of Ceará.

Keyword: diagnosis, multiplex PCR, Mycobacterium ssp, epidemiology

RESUMO

O objetivo do trabalho foi pesquisar Mycobacterium bovis *em lesões sugestivas de tuberculose nas carcaças de bovinos no estado do Ceará, por meio dos testes de diagnóstico bacteriológico e molecular. Entre agosto de 2017 e janeiro de 2019, o Serviço de Inspeção Estadual (SIE) inspecionou 59.512 bovinos; destes, 7,4% (44/59.512) apesentaram lesões sugestivas. Desses animais foram enviadas 68 amostras, das quais 4,5% (31/68) estavam localizadas no pulmão, 2,9% (20/68) nos linfonodos, 2,0% (14/68) no fígado e 0,4% (3/68) na carcaça. Ao realizar o isolamento bacteriológico, 15,9% (7/44) dos bovinos evidenciaram crescimento de colônias nas amostras. Os esfregaços dos isolados foram submetidos à coloração de Zielh-Neelsen e todos eles confirmaram bacilo álcool-ácido resistente. A reação em cadeia da polimerase identificou todos os isolados, 100% (7/7), como* M. bovis*. A associação das técnicas de diagnóstico permitiu identificar a presença do agente no estado, e a análise molecular demonstrou ser uma técnica benéfica no monitoramento da tuberculose bovina, podendo ser utilizada como um método auxiliar no programa de controle e erradicação da tuberculose bovina no estado do Ceará.*

Palavras-chave: diagnóstico, epidemiologia, Mycobacterium *ssp., PCR multiplex*

Corresponding author: amorimqueirozj@gmail.com
Submitted: April 19, 2021. Accepted: December 2, 2021.

INTRODUCTION

Bovine Tuberculosis (TB) is a disease of chronic evolution caused by *Mycobacterium bovis* (*M. bovis*) (Chiu *et al*., 2019), characterized by granulomatous nodular lesions of progressive development, called tubercles, and are commonly seen in the lungs, pleura, adjacent lymph nodes, liver, spleen, and intestine (Vordermeier *et al*., 2012; Bovine, 2019). Bovine Tuberculosis is considered one of the causes of important economic losses to the agricultural sector, which can lead to the death of animals, reduction in weight gain, decrease in milk production and early disposal of animals of high zootechnical value (Pacheco *et al*., 2009; Ahamad *et al*., 2017).

M. bovis has a wide host chain, which can cause zoonotic tuberculosis, being transmitted mainly through the consumption of products of infected animal origin, mainly in regions where food health is deficient. The risk of transmission is greater for occupational workers, due to direct contact with animals, since the pathogen can also be transmitted by aerosols (Bovine, 2020). In this context, sanitary inspection in slaughterhouses is a very important tool for identification suggestive lesions of the disease and tracking outbreaks, aiding the sanitary surveillance of tuberculosis (Souza *et al*., 2016). Some countries such as the United States, Canada, Cuba, Australia, and most of continental Europe have shown that monitoring in slaughterhouses is important to eliminate animals infected with the disease, which can lead to a reduction in bovine tuberculosis in herds (Ayele *et al*., 2004). In Brazil, in 2001, a National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT) was established by the Ministry of Agriculture, Livestock and Supply (MAPA) with the objective of reducing the negative impacts of these zoonoses on human and animal health, (Brasil, 2017). In the state of Ceará, even with the presence of factors that indicate the existence of the disease in certain herds, there is no official data in the state defense agency, and there is a need to intensify the sanitary inspection of herds and slaughterhouses. A study carried out in the state of Ceará using the molecular genotyping technique together with bacteriological isolation confirmed the presence of *M. bovis* in the state, according to the authors, the research can contribute to the creation of a database for further epidemiological studies. (Nascimento, 2014; Ferreira *et al*., 2020).

The diagnosis of bovine tuberculosis can be performed through isolation and identification of the etiological agent in the biological material, known as the "gold standard" (Pacheco *et al*., 2009). However, studies have shown the use of molecular methods such as Polymerase Chain Reaction (PCR) as an auxiliary technique for detecting *M. bovis* DNA in lesions suggestive of tuberculosis, thus allowing for greater accuracy in diagnosis, contributing to the control and eradication of bovine tuberculosis (Cardoso *et al*., 2009; Costa *et al*., 2013; Lorente-Leal *et al*., 2019). Techniques such as multiplex PCR have been shown to be a beneficial tool associated with routine *postmortem* inspection and bacteriological isolation, proving to be a promising method for the health surveillance of bovine tuberculosis (Alzamora Filho *et al*., 2014). Therefore, a study was carried out to identify the presence of *Mycobacterium bovis* in bovine carcasses with characteristic tuberculosis lesions, observed during *postmortem* inspection in slaughterhouses with official inspection service in the state of Ceará, using as complementary diagnosis and confirmatory molecular method associated with the bacteriological test.

MATERIAL AND METHODS

From August 2017 to January 2019, 59,512 cattle were inspected in three slaughterhouses with the State Inspection Service (SIE), located in the municipalities of Maracanaú, Iguatu and Juazeiro do Norte. The lesions suggestive of tuberculosis were collected during the inspection of the bovine carcasses by the Veterinary Medical inspector and placed in sterile universal collectors containing a saturated solution of sodium borate (Na2B2O7•10H2O, 140g/L) as a preservative medium, which preserves mycobacteria for up to 60 days at room temperature (Richards and Wright, 1983). Samples with up to 30 days in saturated borate solution were sent to the Mycobacteriosis Laboratory of the State University of Santa Cruz (Lamvet-Uesc) for isolation and identification of mycobacteria. For bacteriological isolation, lesions suggestive of bovine tuberculosis were decontaminated with 1.5% 1-hexadecylpyridinium chloride (HPC),

inoculated in Stonebrink-Leslie and Lowenstein-Jensen culture media, and incubated at 37°C for up to 90 days and evaluated weekly to identify the growth of colonies suggestive of mycobacteria (Rodriguez, 2005; Brasil, 2008). After bacteriological culture, Ziehl-Neelsen staining was performed on the colonies isolated in the culture media to confirm the staining characteristics of acid-fast bacilli. The isolates characterized as AFB were extracted from DNA by thermal lysis through incubation at 90°C for 30 minutes, followed by centrifugation. The extracted DNA was used to identify *Mycobacterium bovis*, using the molecular multiplex PCR technique, according to the protocol described by Warren *et al*. (2006), including primer designs that are based on the genomic regions of difference of the Mycobacterium tuberculosis complex: RD1, RD4, RD9 and RD12. For the development of the multiplex PCR technique, the genetic material was amplified in a final volume of 25μL with 0.2U of Taq DNA polymerase (HotStarTaq plus DNA polymerase, QIAGEN), 1X PCR buffer, 1X Q buffer, 2.8μL of H2O, 2μL MgCl2, 4μL of dNTP and 0.5μL of each primer. The positive control was obtained at the Mycobacteriosis Laboratory of the State University of Santa Cruz - (Lamvet-Uesc) and for the negative control ultra pure water to assess the appearance of contaminants. The pre-PCR and PCR areas were performed in separate locations. The reaction conditions in a SimpliAmp thermocycler (applied biosystems by life technologies) for DNA amplification were: 95ºC for 5 minutes (denaturation phase); 45 cycles of 94ºC for one minute, 62ºC for one minute and 72ºC for one minute (annealing phase); followed by a final extension phase at 72°C for 10 minutes. Amplification products were separated by electrophoresis (60V/cm) on a 3% agarose gel in 1X TBE buffer and visualized by staining with 10,000-fold diluted SYBR Safe.

RESULTS AND DISCUSSION

Of the 59,512 healthy cattle inspected by *ante mortem* examination, 44 cattle had suggestive lesions. Of these, 68 lesions suggestive of bovine tuberculosis were sent to the laboratory during the *postmortem* examination, obtained during routine slaughter by the official inspection service. The method of diagnosis through sanitary inspection helps to identify macroscopic lesions of bovine tuberculosis in regions with high prevalence and helps to reduce the occurrence of the disease in cattle and transmission to humans and other animals (Corner, 1994). In the present study, the investigation of suggestive lesions through inspection proved to be an efficient tool for identifying *M. bovis* and tracking disease foci in the study region. According to Souza *et al.* (2016), the inspection must be carried out by professionals trained to reduce errors during detection and not cause health damage to the consumer.

The macroscopic lesions suggestive of bovine tuberculosis sent for analysis during the study were located in the lung (31), lymph nodes (20), liver (14) and carcass (3). Data similar to Saidu *et al*. (2015), who inspected 800 cattle in slaughterhouses in Nigeria and observed that the lesions were distributed in the lung, lymph nodes and other tissues. Souza *et al*. (2014), evaluating the frequency of macroscopic lesions in cattle slaughtered in the state of Minas Gerais, observed the occurrence of similar lesions. The data found in the study, in which the highest percentage of lesions was observed in the lung, demonstrates the importance of the respiratory system as an important way of disease transmission, as was also reported by O'Reilly and Daborn (1995). Infection occurs through inhalation of contaminated aerosols, especially in environments with high population density such as dairy herds (Paes and Franco, 2016). Considering the culture as a standard method of tuberculosis diagnosis (Corner, 1994), when performing the bacteriological isolation in the present study, 15.9% (7/44) of the cattle that presented samples with suggestive lesions, exhibited the growth of cream-colored colonies - yellowish, small, rounded, irregular edges, granular surface in Stonebrink-Leslie culture medium and the average time observed for colony growth was 32 days, with a minimum of 23 days and a maximum of 74 days (Fig.1). Alzamora Filho *et al*. (2014) demonstrated similar results in Stonebrink-Leslie culture medium, with an average time to colony appearance of 34 days.

The selection of available culture media, decontamination procedures and incubation conditions are important factors, as they can interfere with the result of bacterial isolation

(Ikuta *et al*., 2016). Yates *et al*. (2017) observed that the number of bacilli in the samples and their storage time until processing can influence the recovery rate of the microbiological culture, which can lead to false negative results. Thus, in the present study, all methods used for processing the culture proved to be efficient.

Samples that showed colony growth were subjected to Ziehl-Neelsen staining, as all smears showed the presence of acid-fast bacilli (AFB). Souza [illegible] (2016) used the Ziehl-Neelsen technique to evaluate 28 samples with lesions in bovine lymph nodes, of which 13.6% demonstrated acid-fast bacilli (AFB), a result similar to the present study.

PCR has high sensitivity and specificity to identify infectious agents, and it is not necessary for the microorganisms to be viable in the biological sample (Haas and Torres, 2016). In the study, multiplex PCR in association with microbiological culture, identified *Mycobacterium bovis* in 7/7 (100%) of the isolates according to the amplification profile of the expected mycobacterial products, of which they present amplification of the fragments related to the RD1 regions (146bp), RD4 (268bp), RD9 (108bp), RD12 (306bp) (Fig.2).

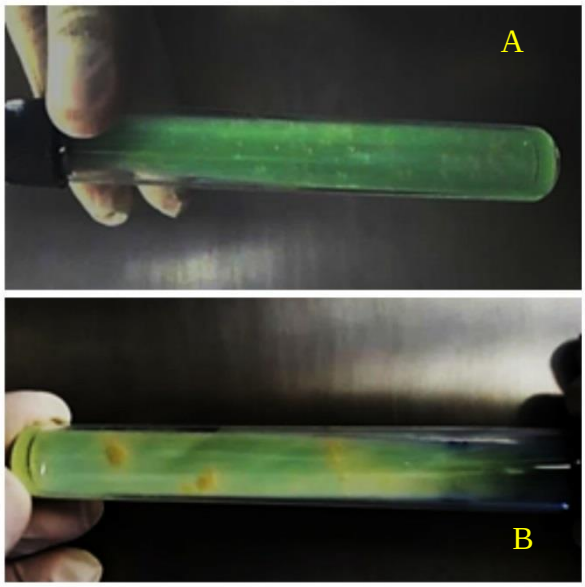

Figure 1. Colonies and Stonebrink-Leslie medium with 32 days of culture (A) and after 60 days of culture (B).

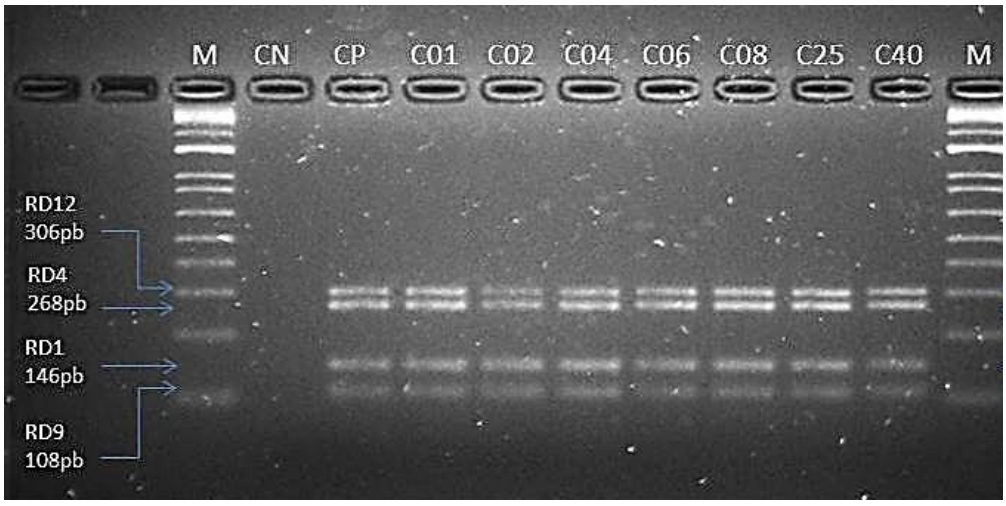

Figure 2. *Mycobacterium bovis* amplification profile by multiplex PCR in bacterial culture isolates. M, molecular weight marker 100bp; CN, negative control (ultra-pure water); CP, positive control of *Mycobacterium bovis*; C01, C02, C04, C06, C08, C25 and C40, bovines positive for *M. bovis*.

The technique based on regions of difference (RD1, RD2, RD4, RD9 and RD12), where the size of the product generated in the amplification corresponds to the absence or presence of regions of difference (RD), were developed for the identification of species of Complex *Mycobacterium tuberculosis*, resulting from multiplex PCR. Allowing a more precise differentiation, making it suitable for routine surveillance and laboratory purposes (Warren *et al*., 2006).

The multiplex PCR method in association with bacteriological culture proved to be valuable for confirming bovine tuberculosis and can be used for screening and differentiation from other mycobacteriosis, as achieved by Asil *et al*. (2013) who used the multiplex PCR technique to detect bovine tuberculosis in the state of South Darfur, Sudan. The use of multiplex PCR as a complementary method for identifying *M. bovis* increases the reliability of the results, ensuring reliability in the diagnosis. Furlanetto *et al*. (2012) in a study carried out in the state of Mato Grosso, using multiplex PCR to identify *M. bovis*, proved to be a beneficial technique that can be used to assist surveillance of bovine tuberculosis in slaughterhouses.

To identify and differentiate between mycobacteria using multiplex PCR, Ramos *et al*. (2018) evaluated 31 (56.4%) samples with lesions suggestive of tuberculosis, and of these, *M. bovis* was identified in 13 (41.9%) of the samples and *Mycobacterium* spp. identified in 18 (58.1%). The authors pointed out that the isolation and identification of *M. bovis* and *Mycobacterium* spp. in bovine carcasses implies that human beings are exposed to the risk of infection. Thus, due to the unpredictable consequences caused by *M. bovis* infection in animal and human health, molecular characterization can support the control and eradication of the disease (Cazola *et al*., 2015).

Considering the sex of the animals, the results of the positive isolates in the PCR presented 0.17% (6/7) for females and 0.08% (1/7) for males. These results are in accordance with the data presented by Ahmad *et al*. (2017) in a study carried out in Nigeria, which observed 226 lesions suggestive of tuberculosis, of which 79 were from males and 147 from females. Admitting what has been described by Acha and Szyfres, (2001) in which dairy females are more likely to acquire the disease than beef cattle, which are slaughtered early, since the females remain longer in the herd and have greater contact during milking.

The presence of *M. bovis* was confirmed through molecular biology in the regions (Iguatu, Jucás and Quixelô) in the Center-South region of Ceará (Fig. 3), an important dairy basin in the state, with emphasis on the municipality of Iguatu that among the 10 cities with the highest production (Ipece, 2018). The presence of the agent in these regions highlights the importance of sanitary surveillance in the municipalities, so that it can carry out continuous control on the properties, seeking to reinforce or even implement the main preventive measures established by the National Program for the Control and Eradication of Brucellosis and Tuberculosis (PNCEBT), thus preventing the risks that the disease can cause to animal and human health.

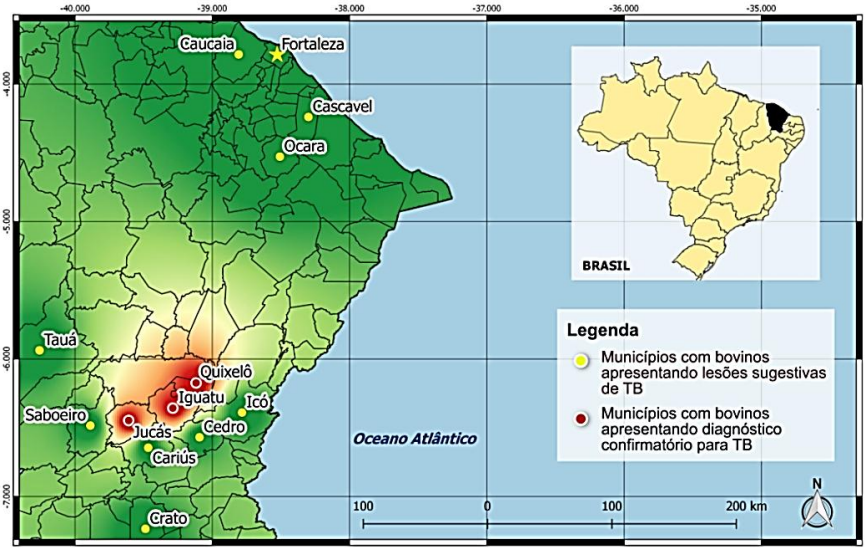

Figure 3. Map of the state of Ceará, highlighting municipalities of bovine origin with lesions suggestive of Bovine Tuberculosis (TB) condemned by the State Inspection Service and municipalities of origin of bovines whose suggestive lesions had a confirmed diagnosis for *M. bovis* by molecular biology.

CONCLUSION

The study showed that the association of *postmortem*, bacteriological and molecular diagnostic methods provided the identification of *M. bovis* in municipalities in the state of Ceará, allowing for a more accurate diagnosis, which could collaborate in an epidemiological survey of bovine tuberculosis, providing information that will help the organs sanitary surveillance in the development of strategies for its control, in addition to the detection of new outbreaks of tuberculosis in the State.

ACKNOWLEDGMENTS

The Coordination for the Improvement of Higher Education Personnel (CAPES) for financial support; to the Agricultural Defense Agency of the State of Ceará (ADGRI) for their collaboration in obtaining information and samples from slaughterhouses in the State of Ceará and to the Postgraduate Program in Animal Science (PPGCA) of UESC for educational support.

REFERENCES

ACHA, P.N.; SZYFRES, B. Tuberculosis zoonótica. In: PERIAGO, M.R. *Zoonosis y enfermidades transmissible comunes al hombre y a los animales*. 3.ed. Washington: Organización Panamericana de la Salud. 2001. 3v. (Publicación Científica y Técnica n.580).

AHAMAD.I.; KUDI, C.Y.; ABDULKADIR, A.I.; SAIDU, S.N.A. Occurrence and distribution of bovine TB pathology by age, sex, and breed of cattle slaughtered in Gusau Abattoir, Zamfara State Nigeria. *Trop. Anim. Health Prod*., v.49, p.583-589. 2017.

ALZAMORA FILHO, F.; REIS, V.M.; FEHLBERG, I.; ALCÂNTARA, A.C. *et al*. Identification of Mycobacterium bovis in carcasses of cattle slaughtered in the state of Bahia, Brazil, by bacteriological and molecular methods. *Braz. J. Vet. Anim. Sci*., v.66, p.1585-1591, 2014.

ASIL, E.T.A.; SANOUSI, S.M.E.; GAMEEL, A. *et al*. Bovine tuberculosis in South Darfur State, Sudan: an abattoir study based on microscopy and molecular detection methods. *Trop. Anim. Health Prod*., v.45, p.469-472, 2013.

AYELE, W.Y.; NEILL, S.D.; ZINSSTAG, J. *et al*. Bovine tuberculosis: an old disease but a new threat to Africa. *Inter. J. Tuberc. Lung Dis.*, v.8, p.924-937, 2004.

BOVINE tuberculosis. OIE, Available in: https://www.oie.int/es/sanidad-animal-en-el-mundo/enfermedades-de-los-animales/tuberculosis-bovina/. Accessed in: 18 Dec. 2019.

BOVINE tuberculosis. OIE, https://www.oie.int/es/sanidad-animal-en-el-mundo/enfermedades-de-los-animales/tuberculosis-bovina/. Access in: 20 de out. 2020.

BRASIL. Ministry of Health. National Manual for Laboratory Surveillance of Tuberculosis and other Microbacteria. Brasília: Ministry of Health, 2008. 436p.

BRASIL. National Program for the Control and Eradication of Animal Brucellosis and Tuberculosis PNCEBT. 2017. Available in: http://www.agricultura.gov.br/assuntos/sanidade-animal-e-vegetal/saudeanimal/ animal-health-programs / brucellosis-and-tuberculosis / tb-1 pncebt.pdf. Accessed in: 12 June 2018.

CAZOLA, D.O.; JORGE, K.S.G.; ZUMÁRRAGA, M.J. *et al*. Identification and genotyping of Mycobacterium bovis in positive cattle in the intradermal test for tuberculosis in Mato Grosso do Sul. *Braz. Vet. Res*., v.35, p.141-147, 2015.

CARDOSO, M.A.; CARDOSO, R.F.; HIRATA, R.D.C. *et al*. Direct detection of *Mycobacterium bovis* in Bovine Lymph Nodes by PCR. *Zoonoses Public Health*, v.56, p.465-470, 2009.

CHIU, L.J.V.; TAUER, L.W.; SMITH, R.L. *et al*. Assessment of the bovine tuberculosis elimination protocol in the United States. *J. Dairy Sci.*, v.102, p.2384-2400, 2019.

CORNER, L.A. *Post mortem* diagnosis of *Mycobacterium bovis* infection in cattle. *Vet. Microbiol.*, v.40, p.53-63, 1994.

COSTA, P.; FERREIRA, A.S.; AMARO, A. *et al*. Enhanced detection of tuberculous mycobacteria in animal tissues using a semi-nested probe-based *real time* PCR. *PLoS One*, v.8,| p.e81337, 2013.

FERREIRA, F.F.; OLIVEIRA, M.L.M.; ALZAMORA FILHO, F. *et al*. Isolation and genotyping of Mycobacterium bovis in suggestive lesions of tuberculosis in cattle slaughtered in the state of Ceará. *Braz. J. Vet. Res*., v.40, p.863-870, 2020.

FURLANETTO, L.V.; FIGUEIREDO, E.E.S.; CONTE JÚNIOR, C.A. *et al*. Use of complementary methods for post-mortem inspection of carcasses with suspected bovine tuberculosis. *Braz. J. Vet. Res.*, v.32, p.1138-1144, 2012.

HAAS, D.J.; TORRES, A.C.D. Aplicações das Técnicas de PCR no diagnóstico de doenças infecciosas dos animais. *Rev. Cien. Med. Vet.*, v.14, p.1-15, 2016.

IPECE informe. Ceará: Instituto de Pesquisa e Estratégia Econômica do Ceará, 2018. Disponível em: https://www.ipece.ce.gov.br/wp-content/uploads/sites/45/2018/09/ipece_informe_128_30_Maio_2018.pdf. Acessado em: 4 out. 2021.

IKUTA, C.Y.; MORATO, F.; SOUZA, G. *et al*. O. Influence of the incubation conditions on culture media to optimize primary isolation of *Mycobacterium bovis*. *Semin. Ciênc. Agrár*., v.37, p.3693-3700, 2016.

LORENTE-LEAL, V.; LIANDRIS, E.; CATELLANOS, E. *et al*. Validation of a Real-Time PCR for the Detection of Mycobacterium tuberculosis Complex Members in Bovine Tissue Samples. *Front. Vet. Sci*., v.6, Art. 61, 2019.

NASCIMENTO, E.T.S. *Epidemiological aspects of bovine tuberculosis in Quixeramobim (CE): dairy basin in the central hinterland of Ceará*. 2014, 71f. Thesis (Master's degree) - *Ceara state University*. Fortaleza, CE.

O'REILLY, L.M.; DABORN, C.J. The epidemiology of *Mycobacterium bovis* in animals and man: a review. *Tuberc. Lung Dis*., v.76, p.1-46, 1995.

PAES, A.C.; FRANCO, M.M.J. Tuberculose em animais de produção. In: MEGID, J.; RIBEIRO, M.G.; PAES, A.C. *Doenças infecciosas em animais de produção e companhia*. Rio de Janeiro: Roca, 2016. v.1, p.512-542.

PACHECO, A.M.; HAMZÈ, A.L.; AVANZA, M.F.B. *et al*. Bovine tuberculosis - case report. *Electron. Sci. J. Vet. Med.*, v.7, n.13, 2009.

RAMOS, M.J.; HEINEMENN, M.B.; FERREIRA NETO, J.S. *et al*. Isolation and identification of *Mycobacterium bovis* in bovines with positive reaction to the tuberculin test in the state of Paraíba, northeast Brazil. *Arq. Inst. Biol.*, v.85, e0842016, 2018.

RICHARDS, W.D.; WRIGHT, H.S. Preservation of tissue specimens during transport to mycobacteriology laboratories. *J. Clin. Microbiol.*, v.17, p.393-395, 1983.

RODRIGUEZ, C.A.R. *System for the detection of bovine tuberculosis outbreaks in the State of São Paulo using molecular and epidemiological methods*. 2005. 86f. Thesis (Doctor in Veterinary Medicine) - Faculty of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP.

SAIDU, A.S.; OKOLOHA, E.C.; GAMAWA, A.A. *et al*. Occurrence and distribution of bovine tuberculosis (*Mycobacterium bovis*) in Slaughtered cattle in the abattoirs of Bauchi State, Nigeria. *Vet. World*., v.8, p.432-437, 2015.

SOUZA, M.A.; BOMONATO, N.G.; SOARES, P.M. *et al*. Frequency of macroscopic lesions in bovine carcasses reacting to the tuberculin skin test. *Arch. Biol. Inst.*, v.81, p.363-367, 2014.

SOUZA FILHO, A.F.; OSÓRIO, A.L.A.; JORGE, S.G. *et al*. Genetic profiles of *Mycobacterium bovis* from a cattle herd in southernmost Brazil. *Semin. Ciênc. Agrár.*, v.37, p.3719-3726, 2016.

VORDERMEIER, M.; AMENI, G.; BERG, S. *et al*. The influence of cattle breed on susceptibility to bovine tuberculosis in Ethiopia. *Comp. Immunol. Microbiol. Infect. Dis.* v.35, p.227-232, 2012.

WARREN, R.M.; PITTUS, N.C.G.V.; BARNARD, M. *et al*. Differentiation of *Mycobacterium tuberculosis complex* by PCR amplification of genomic regions of difference. *Int. J. Tuberc. Lung Dis.*, v.10, p.818-822, 2006.

YATES, G.F.; PRICE-CARTER, M.; BLAND, K. *et al*. Comparison of the BBL mycobacteria growth indicator tube, the BACTEC 12B, and solid media for the isolation *of Mycobacterium bovis*. *J. Vet. Diagn. Invest.*, v.29, p.508-512, 2017.