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Tuberculosis diagnostic methods in buffaloes

Métodos de diagnóstico da tuberculose em búfalos

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Abstract

The low productivity of buffalo herds and condemnation of carcasses in slaughterhouses due to tuberculosis lesions have resulted in increasing economic losses because these animals cannot be treated and must be destroyed by sanitary slaughter. Tuberculosis is a widely distributed zoonosis that affects the beef supply chain of the Brazilian agribusiness economically and socially. Like cattle, buffaloes are sensitive to *Mycobacterium bovis*, which is the main causative agent of zoonotic tuberculosis. Tuberculosis in buffaloes has been reported in several countries, including Brazil. In order to control and eradicate this disease among cattle and buffaloes in Brazil, the Ministry of Agriculture, Livestock, and Supply created the National Program for the Control and Eradication of Brucellosis and Tuberculosis with the main objective of finding a significant number of disease-free herds throughout the national territory using reliable methods. This review summarizes the main data on the history of occurrence of *M. bovis* in Brazilian herds and the diagnostic methods for the disease in buffaloes. Little information is available on buffalo tuberculosis. Due to the increasing population of buffaloes and their economic importance, more studies investigating the occurrence and identification of tuberculosis in this species are clearly needed.
Key words: *Mycobacterium bovis*, isolation, zoonosis, bovine

Resumo

São crescentes as perdas econômicas com baixa na produtividade dos rebanhos bubalinos e a condenação de carcaças em matadouros devido à tuberculose, uma vez que os animais não podem ser tratados e devem ser descartados em abates sanitários. A doença tem impacto econômico e social para a cadeia produtiva do agronegócio brasileiro, além de ser uma zoonose de ampla distribuição. Assim como os bovinos, o búfalo é sensível ao *Mycobacterium bovis*, principal agente da tuberculose zoonótica. A ocorrência da tuberculose nessa espécie tem sido relatada em diversos países, inclusive no Brasil. Para controle e erradicação dessa enfermidade entre os bovinos e bubalinos no Brasil, o Ministério da Agricultura Pecuária e Abastecimento (MAPA) instituiu o Programa Nacional de Controle e Erradicação da Brucelose e Tuberculose (PNCEBT), cujo principal objetivo é, por meio de métodos confiáveis, obter significativo número de rebanhos livres dessas doenças, em todo o território nacional. Esta revisão reuniu os principais dados referentes ao histórico da ocorrência de *M. bovis* nos rebanhos do Brasil e aos métodos diagnósticos da doença em búfalos. Na literatura faltam dados relativos à tuberculose bubalina e com o incremento da criação desses animais e de sua importância econômica, notou-se evidente necessidade de estudos e pesquisas relativos à ocorrência e identificação da tuberculose nessa espécie.
Palavras-chave: *Mycobacterium bovis*, isolamento, zoonose, bovídeos

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Introduction

Tuberculosis is a zoonosis that has been described in cattle slaughterhouses since the 1800s. Before the advent of milk pasteurization, tuberculosis was easily transmitted to the human population from cattle (COSIVI et al., 1995). Even today, 15% of the food produced in developing countries around the world is susceptible to infection from this bacillus (DAVIES, 2006). Tuberculosis in cattle is a public health concern and a potential threat to human health (DAVIES, 2006). Some authors believe that zoonotic tuberculosis is one of the many consequences of the adaptability of the *Mycobacterium* species to different hosts (ANAELOM et al., 2010), although this is controversial (ROTHSCHILD et al., 2001).

Etiology

Mycobacterium Bovis, the main agent of this zoonosis, was once a major problem in developed countries and is currently a problem in developing countries (GRANGE, 2001). Tuberculosis in cattle has been increasingly studied because it easily spreads among herds. In addition, humans are susceptible to the disease-causing agent (O'REILLY; DABORN, 1995).

Epidemiology

The main route of disease transmission among herds is by the introduction of infected animals to areas inhabited by healthy animals (ANAELOM et al., 2010; DAVIES, 2006). Furthermore, the use of shared corrals, drinking and feeding troughs, and the high density of animals in enclosures with poor ventilation could promote the spread of the bacillus (O'REILLY; DABORN, 1995). *M. bovis* affects other animals, in addition to bovines and humans; namely non-human primates, wild boar, deer, ferrets, skunks, badgers, bison, dogs, cats, goats, sheep, and buffaloes (CORNER, 2006; O'REILLY; DABORN, 1995).

In countries with bovine tuberculosis eradication programs, clinical evidence of the disease in cattle is rarely found because the tuberculin test enables a presumptive diagnosis and the infected animals are eliminated before the signs appear (OIE, 2009). Eradication programs exist in European countries, Japan, New Zealand, the US, Mexico, and some countries in Central and South America. Bovine tuberculosis is widespread in Africa, parts of Asia, and some Middle Eastern countries (OIE, 2009).

In Brazil, the Ministry of Agriculture, Livestock, and Supply launched the Brazilian National Program for the Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT) in 2001 with particular guidelines related to cattle and buffaloes. The program's objectives were to reduce the prevalence and incidence of these diseases in the national herds and certify that a significant number of animals were free from these diseases or were being monitored for brucellosis and tuberculosis in order to offer products with low levels of health risks to consumers (BRASIL, 2006). In 2011, Brazilian bovine herds totaled 212,797,824 animals, whereas buffalo herds totaled 1,277,199 (IBGE, 2012). The presence of bovine tuberculosis in Brazil was reported by Kantor and Ritacco (2006), and was considered widespread throughout the country, despite the low rates. From 2000 to 2003, reports indicated a prevalence of 0.8% in the bovine population of the State of Minas Gerais, which represented 5% of the 1,586 herds included in the study (BELCHIOR, 2001). In the State of Rio de Janeiro, the infection rate was 12.7% of the 1,632 heads of dairy cattle studied (KANTOR; RITACCO, 2006). More recent data indicate 0.13% positivity in the 19,631 animals examined in the State of Rondônia, 0.20% positivity in the 2,545 examined in the State of Mata Grosso, 0.07% positivity in the 17,555 animals examined in the State of Bahia, and 0.39% positivity among the 16,045 animals examined in the State of Paraná (FERREIRA, 2010).

Tuberculosis has been diagnosed in the buffalo herds of several countries. In Brazil, the first isolation of *M. bovis* in buffaloes was described in 1971 in São Paulo, even though tuberculosis lesions and positive results in caudal-fold tuberculin tests were reported years before (PORTUGAL et al., 1971). A prevalence of 8% infected animals in the State of Pará and 20% in the State of Amazonas was reported later (KANTOR; RITACCO, 2006).

Clinical signs

In bovines, the disease is usually chronic and debilitating, but can occasionally be acute and rapidly progressive (OIE, 2009). It is characterized by the formation of nodular granulomas that are known as tubers. Any living tissue of the body can be affected, but the lesions are commonly seen in the lymph nodes (particularly of the head and thorax), lungs, intestines, liver, spleen, pleura, and peritoneum. The infection in cattle causes economic loss to agriculture because the animals cannot be treated and must be destroyed by sanitary slaughter. In addition, the yield of the infected animal decreases in terms of milk production as well as weight gain (BRASIL, 2006; DAVIES, 2006).

Diagnosis

Despite the identification of tuberculosis outbreaks in buffaloes of Pakistan, the UK, India, Thailand, Nepal, South Africa, Argentina, and Brazil, the disease has hardly been studied in these animals (GARINE-WICHATITSKY et al., 2010; KANAMEDA et al., 1999; LOPES et al., 2006; MICHEL, 2007, 2008; MOTA et al., 2002; O'REILLY; DABORN, 1995; ROSÁRIO, 2010). Currently, literature discusses lesions that are commonly found and ways of diagnosing tuberculosis in buffalo. Jolles et al. (2005) examined 225 African buffaloes *(Syncerus caffer)* and found lesions in the lungs, lymph nodes of the head and respiratory system, prescapular lymph nodes, and abdominal cavity of the animals that tested positive for the tuberculin test. Michel et al. (2007) found most of the lesions in the respiratory system and lymph nodes of the head in African buffalo. In a similar study of the same species, animals that were responsive to the Interferon Gamma test, which is also used as an indirect diagnostic method for mycobacteria, did not exhibit lesions at necropsy (MICHEL, 2008). Another study on lesions characteristic of tuberculosis in *Syncerus caffer* in wild animals captured for an epidemiological study and that tested positive on the Interferon Gamma Test describes changes in the lymph nodes of the head as well as in the retropharyngeal and bronchial lymph nodes (GARINE-WICHATITSKY et al., 2010).

In Brazil, Freitas et al. (2001) documented lesions in buffaloes *(Bubalus bubalis)* from a slaughterhouse in the State of Pará where strains of *Mycobacterium* spp. were isolated in 60.3% of the 1,735 animals that were examined. Of those that tested positive, 55.1% had lesions in the respiratory system. Furthermore, localized tuberculosis lesions were found in the head and tongue regions (20.1%), carcass (9.6%), abdominal cavity (6.3%), and udder (3.8%). Generalized lesions, which included cases of miliary tuberculosis, were found in 27.9% of the animals examined (FREITAS et al., 2001).

In the State of Pará, lesions were described in the retropharyngeal, mediastinal, and mesenteric lymph nodes in 128 slaughtered buffaloes (RIBEIRO, 2003). In 2011, Ribeiro reported that 0.93% of the carcasses at a slaughterhouse in the metropolitan region of Belém, PA, resulted from slaughter due to hepatic tuberculosis lesions. Mota et al. (2002) followed the slaughter of 14 animals in the State of Amazonas that were considered suspect following tuberculin tests, and they found alterations, mainly in the respiratory lymph nodes. The data collected by Ribeiro (2011) corroborated that presented by Freitas et al. (2001), and both authors attribute the occurrence of buffalo tuberculosis in the Marajó Island to the high rainfall in this location and the

gregarious habits of the buffaloes, which permits airborne horizontal contamination.

The tuberculin skin test (TST), which is an indirect diagnostic method that assesses the cellular immunity of the animal against *M. bovis* (BRASIL, 2006), is the most common procedure used in worldwide programs of bovine tuberculosis control and eradication. The test recommended for bovine cattle under the PNCEBT is also recommended for buffaloes because a standard test specific for this species does not exist. Some studies have described differences in skin reactions in the test in buffaloes compared to cows and when comparing the type of tuberculin used, but no parameters have been established for these species nor are there any reliable results on the subject (FREITAS et al., 2001; KANAMEDA et al., 1999; RIBEIRO, 2003; ROXO et al., 1998).

For the direct diagnosis of tuberculosis, the isolation of mycobacteria and the identification of the etiologic agent are performed in biological material. The collection of material during slaughter occurs by separating the lesions that resemble tuberculosis granulomas, and this is followed by identifying them and freezing them for subsequent isolation (KANTOR, 1988). The causative agent's route of entry determines the site where it can be found, and lesions could be pulmonary or extrapulmonary. In addition to the lesions observed at necropsy, isolation can also be performed on milk samples from suspected animals.

The standard method of diagnosis and isolation of mycobacteria are through cultivation in culture medium (KANTOR, 1988). The media commonly used in laboratories for bacillus isolation are made from eggs. The Löweinstein-Jensen medium, which contains glycerol and asparagine (a nitrogen source), promotes multiplication and development of all mycobacterial species, including *M. tuberculosis* complex, except for *M. bovis*. The Stonebrink medium is made from sodium pyruvate, which provides a carbon source instead of glycerol, and is used especially for the isolation of *M. bovis*. In addition, other media support the growth of mycobacteria very well (KONEMAN, 2008).

For the multiplication of mycobacteria to occur without risk of contamination, decontamination of the sample is necessary before the culture is grown. The *Petroff* method, which is performed with sodium hydroxide and hydrochloric acid, is widely used, although studies have suggested other chemicals that have the decontaminant effect (BALIAN et al., 2002). The main concern about these is related to the toxicity of these substances to the infectious agent.

The preservation of clinical samples with sodium carbonate, cetylpyridinium chloride, or sodium borate can be effective, especially in regions with limited access to laboratories and/or the refrigeration of the samples (BOBADILLA-DEL-VALLE et al., 2003). After the growth of mycobacteria in culture medium, the presence of alcohol-acid resistant bacilli (Mycobacteria characteristic) is identified with optical microscopy in slides stained with the Ziehl-Neelsen technique (KANTOR, 1988). Other cultivation methods have been tested in order to enhance the diagnostic techniques for mycobacterial species. Researchers have shown that the use of a Middlebrook 7H11 thin layer agar plate produces a marked difference in the speed of observation of the colonies of *M. bovis* and *M. tuberculosis* (MARCONDES et al., 2006). Apparently, in a relatively short period (between 12 to 25 days), it is possible to observe the growth of mycobacteria, which is an advantage over commonly used media, especially when it is associated with the use of polymerase chain reaction (PCR) for detection of microcolonies (ROSÁRIO et al., 2014).

A technique that has been poorly studied is the multiplication of the bovine bacillus in a culture of macrophages for subsequent amplification by PCR. This technique, which was described by Ritelli et al. (2003), allows for a definitive diagnosis within 48

to 72 h after the collection of a clinical sample when associated with PCR.

For the identification of the bacilli, biochemical analyses and molecular biological tests are performed. Researchers have used PCR techniques and *Mycobacterium* typing with the spoligotyping technique following the isolation of the agent. The PCR technique allows for the amplification of genes with nucleotide sequences (primers), which ensure the specificity of the reaction through hybridization with the DNA target sequence from the sample, and DNA amplification is then performed (TELENTI et al., 1993). After this step, the amplified DNA fragments are placed in an electrophoresis gel that is stained with ethidium bromide or labeled primers for visualization of the DNA bands.

As a complement to the identification techniques, researchers have been studying the molecular principle of spoligotyping, especially for *M. bovis* typing, through the presence or absence of the known spacers in its sequence. This technique is performed after PCR with the product of the DNA amplification that resulted from the mycobacterial agent. The amplified products have spoligotypes that, when identified by the technique, enable the detection and differentiation of strains of the *M. tuberculosis* complex (RODRIGUEZ et al., 2004). Some spacers that are already known among the mycobacteria genes allow for the comparison, differentiation, and epidemiological studies of these agents (ZUMÁRRAGA et al., 1999a, 1999b).

In a survey that was conducted on 15 buffaloes that were slaughtered in the State of Pará and that had suggestive lesions in their lymph nodes, the isolation of alcohol-acid resistant bacilli in 11 animals was performed, and all were identified with *M. bovis* as a result of PCR amplification with the JB21-JB22 primers. Amplification of the mycobacteria DNA with these *primers* resulted in the identification of 13 samples from animals with macroscopic lesions in slaughterhouses (from the states of Pará and São Paulo), and 12 of these 13 animals tested positive on the tuberculin Cervical Comparative Test. The isolated agent of these animals was *M. bovis* (BARBOSA et al., 2012).

Mycobacteria samples for molecular studies can be frozen at -20 °C or in cards for the rapid analysis of nucleic acids. These cards are a kind of filter paper that can conserve samples and their respective characteristics; they inactivate the bacteria, thus facilitating its handling and transport to room temperature (GUIO et al., 2006; WHATMAN PROTOCOL, 2012).

Final Considerations

There are gaps in the laboratory diagnosis of tuberculosis in buffaloes than that performed in cattle. Some authors have suggested that a longer microbiological diagnosis might be required for buffaloes; however, in practice, research has indicated no difference between cattle and buffaloes. Roxo (2012) analyzed bacillus multiplication from buffalo samples and observed a mean duration of 45 days (from 25 to 69 days), which coincided with the degree of macroscopic lesions that were observed in the samples.

When analyzing the history of tuberculosis as a human and animal disease, its zoonotic potential, and the difficulty in isolation of the mycobacterium as a diagnostic method, especially in buffaloes, the need for more studies and research in the respective area is clear.

We emphasize the importance of diagnosing tuberculosis in animals as one of the major factors influencing the prevention of zoonosis. This disease is therefore a public health concern considering that people could consume milk, meat, and other products of animal origin that are infected and not diagnosed. In addition, the occupational nature of the disease should be considered.

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