

SHORT REPORT

Bacteriological and molecular detection of *Mycobacterium bovis* in cattle with inconclusive results to intradermal tuberculin tests

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SUMMARY

A dairy herd (77 cows) from Rio de Janeiro, Brazil, with a history of tuberculosis infection was tested by a comparative cervical test (CCT). Seventeen cows were reactive and seven were inconclusive (swelling ≥ 2.0 mm and ≤ 3.9 mm, respectively). All of these 24 cows were slaughtered and necropsied; samples from lungs and lymph nodes were collected for multiplex polymerase chain reaction (PCR) and culturing. Infection was confirmed in 23/24 (95.8%) of the slaughtered animals (five by culturing, four by PCR, and 14 by both tests). All cows with inconclusive results at CCT were confirmed as infected. Although slaughter of inconclusive reactor cows is not mandatory in many countries, our study provided evidence to support the slaughter of these cows, at least during an outbreak.

Key words: Bovine tuberculosis, comparative cervical test, inconclusive, *Mycobacterium bovis*, PCR.

Bovine tuberculosis (bTB) is an important zoonosis with serious implications for both animal production and public health. Despite considerable efforts in several countries to eradicate this disease, it remains prevalent. Although intradermal tuberculin tests (ITTs) lack both sensitivity and specificity for detection of bTB, they are still used worldwide [1]. The cervical intradermal test (CIT) involves intradermal inoculation of a purified protein derivative of *Mycobacterium bovis* (PPD_{bov}). After 72 h, the injection site is observed for evidence of a local

hypersensitivity reaction. Alternatively, a comparative cervical test (CCT) using a purified protein derivative of *M. avium* (PPD_{av}) may be performed concurrently with PPD_{bov} [2].

It has been recommended [2] that when the induration of the skin measurements is between 2.0 and 3.9 mm (CIT) or the difference between both PPD (PPD_{bov} minus PPD_{av}) reactions is between 1.0 and 3.9 mm (CCT) the animal is considered an inconclusive reactor (IR), and may remain in the herd for 42–60 days pending confirmatory tests. However, it should be noted that these animals represent a potential source of infection for both herd mates and humans.

Although the exact mechanisms of the cellular-mediated response that leads to inconclusive results remain to be clarified, several potential reasons have

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Table 1. Relationship between the cervical comparative test (CCT) and post-mortem direct detection of *Mycobacterium bovis* (PCR and bacterial culture) in 23 dairy cows

Test	PCR	Culture	PCR + culture	Total
CCT positive	3	3	10	16
CCT inconclusive	1	2	4	7
Total	4	5	14	23

One slaughtered CCT-positive cow was not confirmed by either culture or PCR.

been reported. For example, truly non-infected animals that were exposed to environmental mycobacteria could weakly react to the CCT. Moreover, PPD quality, concomitant infections, or misinterpretation of the results could lead to doubtful results [3]. In some cases, animals are sensitive to both PPD_{bov} and PPD_{av} [1].

The purpose of this study was to evaluate, during an outbreak, whether cattle designated IR (based on intradermal tests), were truly infected with *M. bovis*.

Animals. A dairy herd with 77 adult (aged >6 months) crossbred Holstein × Gir cows in Rio de Janeiro, Brazil, that had been considered TB-free for the preceding 5 years had two ITT-positive cows following a routine test conducted after the introduction of newly acquired cows to the herd. After a 90-day period, a CCT was conducted on all 77 animals. Both reactive and IR cows were slaughtered and necropsied. Samples of lungs and mediastinal and bronchial lymph nodes were processed for bacterial culture and polymerase chain reaction (PCR).

Intradermal tests. The test was conducted in accordance with the regulations of the Brazilian Department of Agriculture [4]. The CCT was performed by injecting 0.1 ml PPD_{bov} (*M. bovis* strain AN5, 1 mg protein/ml; Instituto Biológico, Brazil) at the cervical area of each cow and 0.1 ml PPD_{av} (*M. avium* strain D4, 0.5 mg protein/ml; Instituto Biológico) ~20 cm from the PPD_{bov} inoculation site. After 72 h, the sites were measured with calipers and the cow was considered reactive if the difference between the thicknesses of both inoculation sites was >4.0 mm, and IR if that difference was between 2.0 and 3.9 mm.

Bacterial culture. Samples of lungs and mediastinal and bronchial lymph nodes were collected (from each

animal) aseptically, sliced into small pieces, and separately homogenized. Almost 5 g of each tissue sample were macerated with ground glass and suspended with 20 ml of 0.85% sterile saline. From each tissue, 1 ml of the supernatant was collected to compose a 3-ml pool. Samples were decontaminated by four methods: NaOH 4.0% (Petroff Method), H₂SO₄ 12%, CPC 1.5% (cetyl-pyridinium chloride), and NALC (*N*-acetyl-L-cysteine-NaOH). Each pellet was resuspended in 0.5 ml sterile saline (0.85%), and 0.2 ml of the solution was inoculated onto two slopes of a solid, egg-based medium (Lowenstein–Jensen with 0.5% pyruvate). Cultures were incubated at 37 °C and observed once weekly for 12 weeks. Suggestive colonies were confirmed by PCR (described below).

PCR. The suspension of tissues were processed for a multiplex PCR that used primers from the IS6110 sequence of the *M. tuberculosis* complex and primer RvD1Rv2031c from *M. bovis*. Primers used were: INS1 (5'-GTGAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAGGCGTCGGTGACAAA-3'), JB21 (5'-TCGTCCGCTGATGCAAGTGC-3'), and JB22 (5'-CGTCCGCTGACCTCAAGAAAG-3') (Invitrogen, USA) [5].

From the 77 cows of the herd, 17 (22.1%) were positive by CCT while seven (9.1%) presented inconclusive results. Therefore, 24 cows were slaughtered and necropsied. At necropsy, seven animals (including one that was inconclusive by CCT) presented macroscopic characteristic lesions of bTB, i.e. granulomas with necrosis and caseosis or calcification in its centre in the lungs.

Considering the standard methods, i.e. bacterial culture and PCR, infection was confirmed in 23/24 (95.8%) of the slaughtered animals, which unequivocally confirmed the aetiology of the outbreak. In relation to the results of the direct tests, five cows were diagnosed by culture, four by PCR and the remaining 14 by both tests. All seven cows that were IR by CCT were confirmed infected – two by culture, one by PCR and the remaining four by both tests (Table 1).

Some cows were diagnosed by PCR only (they were culture negative). It is of note that PCR has been described as an important tool for diagnosis of bTB, since it is a rapid and sensitive method, capable of a positive response with non-viable mycobacteria [3]. The m-PCR used in the present study had 88.24% sensitivity and 100% agreement with microbiological methods [5]; therefore, this PCR test, as well as bacterial culture, was regarded as a standard method.

Concurrent bacterial culture and PCR enhanced the efficacy of making a diagnosis of TB. In the present study, both tests had similar sensitivities; bacterial culture identified 19/23 infected animals (sensitivity 82.6%), whereas PCR identified 18 animals (sensitivity 78.3%), but not the same cows. Therefore, molecular methods for identification of *M. bovis* (e.g. m-PCR), combined with bacterial culture, augmented the sensitivity and specificity of TB diagnosis, consistent with previous reports [3, 5].

The official recommendation of the OIE, as well as regulations of many countries, including Brazil, does not require that IRs be slaughtered before they are retested, which could require 42–60 days or more [2, 4]; however, this could allow an infected IR to disseminate the infection to herd mates or humans. Although in the EU, regulatory authorities can adjust the interpretation of the tests during an outbreak [6], the OIE and many other countries do not consider different criteria for outbreaks. Additionally, although the risk of transmitting TB via the milk of tuberculous cows has been extensively described [1], in many countries there is no formal policy regarding the disposition of milk from IR cows during this period, except for the EU, where their milk is withheld from the human food chain.

In the present outbreak, 7/77 cows had inconclusive results; this represents 9.1% of the herd, with all of them confirmed as infected by standard methods (culture and/or PCR). Although few reports regarding IR animals have been conducted, a recent study performed in Ireland [7] reported that 11.8–21.4% of the IR animals that were slaughtered before a retest were confirmed as positive at post-mortem examination (e.g. histopathology or bacteriological culture). Additionally, negative CCT animals in a herd with IR animals were more likely to become reactive as they live and share almost the same area and fomites [8], therefore reinforcing the risk of maintaining those animals in the herd while waiting for confirmative test results. In the USA [9] and in Brazil [4], IR animals are kept in the herd for up to 60 days until confirmatory tests can be conducted. In those countries, slaughter is required only after one positive or two additional inconclusive results. By contrast, in England, animals are removed after the second inconclusive result [10]. Based on these findings, it is suggested that, at least during an outbreak, cattle with inconclusive reactions at intradermal tests have a high probability of being infected and should be immediately slaughtered to

avoid dissemination of the infection among other animals and humans.

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DECLARATION OF INTEREST

None.

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