Short Communications

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Mycobacterium bovis detection from milk of negative skin test cows

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MYCOBACTERIUM bovis is the bovine tuberculosis (bTB) agent, which is still an important infection in bovine herds. Apart from this, bTB is also a zoonotic disease, and human beings can be infected by the consumption of non-pasteurised milk and its derivatives (Michel and others 2010). Hence, bTB is still endemic worldwide (Humblet and others 2009).

The single intradermal test (SIT) is a worldwide diagnosis test, which is known to lack from both sensitivity and specificity (de la Rua-Domenech and others 2006). In order to augment the specificity of this test, a single intradermal comparative cervical tuberculin (SICCT) test is often recommended for animals with inconclusive results at an SIT (Medeiros and others 2010). Although the SICCT test represents an advance in terms of increase in specificity, few advances were made in order to augment the sensitivity of intradermal tests, referred to be 80–93 per cent (de la Rua-Domenech and others 2006). Another important limitation of the intradermal tuberculin test (ITT) refers to anergy, when animals in late stages of the disease present undetectable cellular immune response and are consequently false-negative to the intradermal tests (Medeiros and others 2010).

Even during an outbreak, once a cow is classified as negative by ITT, there is no recommendation for additional tests. Since ITT-negative animals are not slaughtered, additional investigation of these cows may be impaired by the difficulty in obtaining samples from live animals. Nevertheless, that assumption would only be correct if ITT was referred as 100 per cent sensitive, which is far from reality. Therefore, that strategy usually leads to the misdiagnosis of false-negative animals in the herd, jeopardising the control of the infection.

The purpose of this study was to investigate the possible shedding of *M bovis* by the milk in SICCT-negative test cows during an outbreak of bTB.

Animals. A dairy herd with 77 adult (>six months) crossbreed Holstein-Gir cows in Rio de Janeiro, Brazil, that had been considered tuberculosis (TB)-free for the last five years presented two SICCT-positive test cows on a routine test conducted after the introduction of newly acquired cows. After a 90-day period, SICCT was conducted on all the 77 animals. This research was submitted to the research and postgraduate committee (CEPA-UFF), protocol 0234/12.

Intradermal tests. An SICCT test was conducted in accordance with the regulations of the Brazilian Department of Agriculture (Brasil 2006). It was performed by injecting 0.1 ml of bovPPD (*M bovis* strain AN5, 1 mg protein per ml; Instituto Biológico, São Paulo, SP, Brazil) in the cervical area of each cow, and 0.1 ml of avian PPD (*Mycobacterium avium* strain D4, 0.5 mg protein per ml; Instituto Biológico) approximately 20 cm from the bovPPD inoculation. After 72 hours, the site was measured with callipers and the cow considered reactive if the difference between the thicknesses of both sites of the inoculation were >4.0 mm, and inconclusive if that difference was between 2.0 and 3.9 mm.

Bacterial culture. Milk samples were obtained from all the eight lactating cows that were SICCT-negative at the time of the collection. Each sample was mixed with 0.1 per cent of Tween 80. Five millilitres aliquots of each sample were decontaminated by three different methods: 4 per cent NaOH (Petroff Method), 12 per cent H_2SO_4, 1.5 per cent cetylpyridinium chloride, according to Medeiros and others (2012). Samples were centrifuged (4500 g/16 minutes); sediment was inoculated onto two slopes of a solid, egg-based media (Lowenstein-Jensen with 0.5 per cent pyruvate). Cultures were incubated at 37°C and observed weekly for 12 weeks. Suggestive colonies were confirmed by the same PCR protocol described below.

PCR. Milk samples were tested by m-PCR that employed two sets of primers simultaneously: the *RvD1Rv2031c* (500pb) specific for *M bovis* and *IS6110* (245bp) sequence present in all MTC species. Primers employed were: INS1 (5′-GTGAGGGCATCGAGGTGGC-3′) and INS2 (5′-GCGTAGGCGTCGGTGACAAA-3′) for MTC, JB21 (5′-TCGTCCGCTGATGCAAGTGC-3′) and JB22 (5′-CGTCCGCTGACCTCAAGAAAG-3′) for *M bovis* (Invitrogen) (Figueiredo and others 2012).

SICCT tests revealed 17 (22.1 per cent) positive cows, while seven presented inconclusive results (9.1 per cent). Five (62.5 per cent) out of the eight milk samples collected from negative SICCT test cows were positive to *M bovis*; four of them only by PCR, and only one by culture (Table 1).

After a period of 90 days, a new SICCT test was performed and two of those cows (001 and 049) were positive, while two others (019 and 023) were inconclusive; those four cows were slaughtered and necropsied according to Brazilian law; and two of them (001 and 023) presented macroscopic characteristic lesions of bTB, that is, granulomas with necrosis and caseosis or calcification in its centre in the lungs. Despite the positive results on milk, the remaining four cows were not slaughtered.

Although *M bovis* has been extensively recovered from milk of cows from naturally infected herds (Medeiros and others 2010), there is a lack of studies directed towards the examination of the SICCT-negative test cow's milk. In India, Srivastava and others (2008) isolated the bacterium from six out of 154 (3.9 per cent) milk samples; from these samples, four were from SICCT-negative test cows.

TABLE 1: Results of *Mycobacterium bovis* direct detection (PCR and bacteriological culture) from milk samples obtained from eight cows that were negative to intradermal tuberculin tests in a naturally infected herd

Animal	Milk PCR	Culture
001	Positive	Negative
014	Negative	Negative
019	Positive	Negative
020	Positive	Negative
023	Positive	Negative
027	Negative	Negative
049	Negative	Negative
054	Negative	Positive

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PCR has been described as an important tool for the diagnosis of bTB, since it is a rapid and sensitive method, diagnosing even samples with non-viable mycobacteria (De la Rua-Domenech and others 2006, Medeiros and others 2010). Bacteriological culture and PCR applied in parallel enhanced the efficacy of direct diagnosis of tuberculosis. PCR for the detection of *M bovis* DNA in milk samples has been more commonly reported; in Brazil, Figueiredo and others (2012) reported the identification of specific *M bovis* DNA in 12 per cent of the milk samples obtained from SICCT-positive test cows. In Argentina, Zumárraga and others (2012) reported positive results employing PCR in milk samples from bulk tanks, from TB-suspected and also from certificated TB-free herds. In those samples, no culture was obtained.

In conclusion, it has been demonstrated that ITTs were not enough to correctly identify all the infected cows of the herd; therefore, during an outbreak situation, additional tests of samples collected from SICCT-negative cows could collaborate for an adequate control of the disease in the herd, reducing the possibility of affecting human beings by the consumption of raw contaminated milk.

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Competing interests None.

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