DETECTION OF MYCOBACTERIUM BOVIS DNA IN NASAL SWABS FROM TUBERCULOUS CATTLE BY A MULTIPLEX PCR

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ABSTRACT

Detection of tuberculosis in cattle relies on the intradermal tuberculin test (ITT), but a definitive diagnosis requires identification of the pathogen after the animal is slaughtered. DNA in nasal swabs from 50 cows was analyzed by m-PCR, targeting for the RvD1-Rv2031c and IS6110 sequences. M. bovis was identified in two of 34 tuberculous cows (5.9%). The use of mPCR of nasal swabs as an in vivo diagnostic tool for bovine tuberculosis is suggested.

Key words: Mycobacterium bovis, multiplex-PCR, nasal swab, bovine tuberculosis

Bovine tuberculosis (BT) is a major problem in many countries, and poses economic and public-health hazards. In Brazil, despite an official control program based on a test-and-slaughter policy, the reported prevalence of this disease in cattle ranges from 0.7% to 2.8% (17, 1), and economic impact studies suggest a 25% impairment of production (16).

Bovine tuberculosis infection in cattle is usually diagnosed in the live animal based on delayed hypersensitivity reactions (intradermal tuberculin tests), which may lack both sensitivity and specificity (20). However, a definitive diagnosis is still established by isolation and identification of the etiological agent (Mycobacterium bovis) from lymph nodes or lungs, obtained during necropsy or at slaughter, using a combination of traditional culture and biochemical methods; this is considered as the “gold standard method”. These methods are slow, cumbersome, unreliable, and time-consuming (it may take more than 4 weeks to grow the microorganism, and an additional 2 weeks to identify it). Several alternative approaches have been attempted for the rapid and specific diagnosis of tuberculosis (5), but the molecular methods, especially polymerase chain reaction (PCR) assays, are the most promising.

A number of PCR-based protocols have been developed for the detection of mycobacteria belonging to the Mycobacterium tuberculosis complex (MTC), which comprises five species (Mycobacterium tuberculosis, M. bovis, M. africanum, M. microti and M. canetti). (21, 24). The most common protocols use primers that amplify segments of the IS6110 genomic insertion characteristic of MTC (4, 6). Specific identification of M. bovis strains by PCR focuses on the amplification of a 500-bp DNA fragment located inside a genomic 4,999-bp region at the 3’ end of a putative gene called RvD1-Rv2031c, which is not present in other mycobacterial species (18). Although these DNA tests are more reliable and much more rapid than traditional identification methods, tissue samples are still required, and the tests are still limited to the post-mortem diagnosis of the infection, despite some studies regarding their use on milk samples (25, 8).
In this study, the aim was to standardize a multiplex PCR-based method for the simultaneous detection of mycobacteria belonging to MTC and the specific identification of \textit{M. bovis}, using DNA samples extracted from nasal swabs collected from live cows. A total of 50 adult cows from a dairy herd with a previous history of bovine tuberculosis, including clinical cases, from Macaé city (Rio de Janeiro state, Brazil), were tested by the cervical comparative intradermal tuberculin test (ITT) with PPD (purified protein derivative) according to official standards (3). In parallel, samples of nasal mucus were collected using sterile swabs, and submitted to both microbiological culture and m-PCR.

All 34 ITT-reactive animals (68\% of the total cows examined) were slaughtered; the lungs and lymph nodes were removed and processed for bacteriology according to the OIE Terrestrial Manual (15). Briefly, after decontamination by the Petroff method, tissue samples from the lungs and lymph nodes, and nasal swabs were inoculated on Lowenstein-Jensen and Stonebrink agar slopes, and the tubes were incubated at 37°C for up to 12 weeks. The presence of \textit{M. bovis} and other mycobacteria belonging to MTC in the nasal mucus was investigated by a single-step multiplex PCR (m-PCR), using two sets of primers previously described (8) that target simultaneously the \textit{RvD1-Rv2031c} (specific for \textit{M. bovis}) and IS6110 (present in all MTC species) genomic sequences, but that to date had not been used together in a single m-PCR assay. DNA was extracted from the nasal swabs by a modification of a QIAamp Blood and Tissue Kit (Qiagen). The bacterial pellet was suspended in 180 µl of 20 mg/mL lysozyme in 20 mM Tris-HCl, pH 8.0; 2 mM EDTA; and 1.2% Triton, and incubated for 30 min at 37°C prior to the proteinase K treatment, in order to improve the process of bacterial lysis. DNA eluted from the QIAamp Mini spin columns was concentrated by precipitation with absolute ethanol at -80°C. m-PCR was performed in a reaction mix (50 µL) containing 5 µl of 10× PCR buffer (Invitrogen), 200 µM dNTP (GE Healthcare), 2.5 U of recombinant \textit{Taq} polymerase (Invitrogen), 2.0 mM MgCl\textsubscript{2}, 5 µL of DNA, and 0.2 µM of each primer (JB21 (5'-TCGTCCGCTGAGTCAGGC-3') and JB22 (5'-CGTGAGCTAAGTCAGGC-3'), targeting for the \textit{RvD1-Rv2031c} sequence; and INS1 (5'-CGTGGAGGCATCAGGCGGC-3') and INS2 (5'-CGTGGAGGCATCAGGCGGC-3'), targeting for the IS6110 region. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems), using an initial denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 68°C for 1 min, and elongation at 72°C for 1 min. Cycling was completed by a final elongation step at 72°C for 7 min. The reaction products were resolved by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining (10 µg/mL) and examination under UV light.

No mycobacterial growth was observed on agar slopes inoculated with nasal swab samples collected either from ITT-reactive or ITT-negative cows. However, mycobacterial colonies were observed in cultures from lungs or lymph node samples isolated from 17 of 34 PPD-positive cows (50\% of the total of positive cows). In parallel, nasal swabs were searched for the presence of mycobacteria by m-PCR. DNA was extracted from nasal swabs collected from 34 ITT-reactive and 16 ITT-negative cows, using a modification of a QIAamp Blood and Tissue Kit (Qiagen) that was devised to improve bacterial lysis and concentrate DNA. The nucleic acids isolated from all samples using the above-modified procedure showed high quality in terms of integrity and purity, and were suitable to be used as templates in the m-PCR. The 500 bp fragment specific for \textit{M. bovis} and the 245 bp fragment diagnostic for MTC were simultaneously observed in two of 34 (5.9\%) m-PCR reactions performed that used nasal swabs containing DNA from ITT-reactive cows (Figure 1, lanes 1 and 2). Importantly, neither the 500 bp band nor the 245 bp band was found as an m-PCR reaction product when swabs from ITT-negative cows were tested (results not shown).

\textit{M. bovis} has been recovered from nasal exudates collected from cattle in naturally infected herds by using conventional culture techniques (4, 12, 13). In these reports, recovery efficiencies varied from 8.7 (4) to 28.5\%, when only ITT-reactive animals were assessed (13), regardless of the difficulty

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of the procedure. The efficiency is low because the presence of
10-100 viable organisms in the sample is required for a positive
result, a condition attained only in advanced stages of the
disease (2).

![Image](image_url)

**Figure 1.** Identification of *M. bovis* in nasal swabs by m-PCR.
DNA obtained from nasal swabs of 34 reactive and 16 non-
reactive animals was used as templates for simultaneous
amplification of the *RvDI*-Rv2031c and the IS6110 sequences.
PCR products were resolved by electrophoresis on 1.5%
agarose gel and stained with ethidium bromide (10μg/mL).
Lane M: 100 bp DNA ladder (Fermentas Life Sciences); lanes
1-2: DNA from ITT and bacteriological-positive animals; lane
3: positive control (DNA from *M. bovis* IP); lane 4: negative
control (PCR without DNA template). Arrows indicate the
position of the 500 bp (diagnostic for *M. bovis*) and the 245 bp
(diagnostic for MTC members) fragments.

In one study using PCR-based methods, the presence of
MTC-group species in nasal exudates of ITT-reactive animals
was detected in 26% of tested samples (22). Some studies have
reported detection rates of 50 or 58% (13, 23). These rates
seems to be rather low, because some PCR techniques can
detect *M. bovis* using as little as 5 fg of DNA, which
 corresponds to the amount of nucleic acid in a single
mycobacterial genome (7). These lower-than-expected results
were probably caused by limitations in the current PCR
protocols for detection of mycobacteria in nasal exudates, such
as intermittent shedding, inefficient DNA extraction, or the
presence of PCR inhibitors in the samples (5).

In the present study, none of the nasal-exudate samples
from 34 ITT-reactive cows was found to be positive for the
growth of *M. bovis*. Furthermore, 2 of 34 nasal-exudate
samples (5.9%) were positive for m-PCR, a more sensitive
and specific method than culturing (13, 25, 26). These figures are
lower than those previously obtained by using culture or PCR-
based methods for the presence of *M. bovis* in nasal exudates
(4, 13, 13, 22, 23). These low figures may be a consequence of
the small number of viable bacteria in swab nasal samples,
because the growth of the etiological agent was observed in
cultures of lung and lymph-node samples from 17 of the cows,
using the same procedure.

It has been well documented that in cattle experimentally
infected with *M. bovis*, after every infection there is a lag
period during which the microorganism cannot be isolated
from nasal mucus (11, 10, 14). In one study, all the
experimentally infected animals shed *M. bovis* in nasal mucus
(14); however, failure of some experimental animals to shed
mycobacteria has also been reported (10, 11). Importantly,
differences in the shedding profiles were observed, where those
animals shedding *M. bovis* in nasal exudates were classified as
intermittent or otherwise as persistent shedders. It also appears
that the overall level of shedding increases during the first four
weeks after exposure and then begins to decrease (10), but the
bacteria could still be detected for many weeks and in some
cases, for several months.

In conclusion, we have successfully used an m-PCR assay
to detect *M. bovis* in nasal exudates of naturally infected cattle,
as previously reported by others (13, 22, 23). Indeed, Vitale et
al. (1998) reported high specificity and positive predictive
value in the detection of MTC in nasal swabs by PCR, and
Romero et al. (1999) demonstrated that nasal-mucus samples
give better results for the in vivo PCR-based detection of the
microorganism than other body fluids such as blood or milk.
However, all these previous reports used primers to detect
MTC species, and identification of *M. bovis* was presumptive. The mPCR used in this study has the advantage that it detects *M. bovis* specifically, but also simultaneously identifies the presence of *M. bovis* and other non-*M. bovis* mycobacterial species belonging to MTC. Although limited by the natural evolution of the infection, because shedding of mycobacteria in nasal mucus is required, the m-PCR described here could be used as an effective and highly specific ante mortem ancillary method for surveillance of bovine tuberculosis in herds, if a periodic sampling scheme is followed; or as a confirmatory method for animals with inconclusive intradermal test results, thus assisting the bovine tuberculosis control and eradication program.

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REFERENCES


