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Molecular Techniques for Identification of Species of the *Mycobacterium tuberculosis* Complex: The use of Multiplex PCR and an Adapted HPLC Method for Identification of *Mycobacterium bovis* and Diagnosis of Bovine Tuberculosis

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1. Introduction

Mycobacterium bovis is a member of the *M. tuberculosis* complex (MTC), a group of species (*Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti*) with a high genetic homology. *M. bovis* is the causative agent of tuberculosis in a range of animal species and humans, with worldwide annual losses to agriculture of \$3 billion. The human burden of tuberculosis caused by the bovine tubercle bacillus is still largely unknown. *M. bovis* was also the progenitor for the *M. bovis* bacillus Calmette–Guérin vaccine strain, the most widely used human vaccine. Garnier et al. (2003) described the 4,345,492-bp genome sequence of *M. bovis* AF2122/97 and compared it with the genomes of *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Strikingly, the genome sequence of *M. bovis* is >99.95% identical to that of *M. tuberculosis*, but deletion of genetic information has reduced the genome size.

Bovine tuberculosis (BTB) is a major infectious disease of cattle in many countries. Although cattle are the main host and reservoir of this chronic infection, other mammalian species, including humans, are also susceptible to *Mycobacterium bovis* (Romano et al., 1996). Considering that more than 94% of the world population lives in countries where the

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control of bovine tuberculosis is limited or absent (Vordemeier, 2006), there is a consensus about the risks regarding human health.

Humans can develop latent TB infection, active TB or reactivation of latent TB infection. In veterinary medicine, the distinction between latent TB infection and active TB disease is not as important as it is in human beings, since in both cases the animal must be slaughtered. According to the National Control Program implemented in Brazil, treatment of reactive animals is not allowed, and all reactive animals must be slaughtered (Brasil, 2004).

Zoonotic TB is a recognized public health threat in the developing world. In some countries, control measures against bovine tuberculosis are limited or nonexistent (Cosivi, 1998; Thoen, 2006). Human infection by *M. bovis* can occur by the aerogenous route, ingestion of infected milk (WHO, 1994), or, less frequently, by contact with mucous membranes and broken skin. The disease caused by *M. bovis* is clinically, radiologically and pathologically indistinguishable from that caused by *M. tuberculosis*, while, differently from the typical tuberculosis and due to the infection route, the non-pulmonary presentation is most frequent (Grange, 2001). Considering the standard treatment for *M. tuberculosis* infection, the lack of differentiation between *M. bovis* and *M. tuberculosis* is a problem. *M. bovis* is naturally resistant to pyrazinamide, a drug that is frequently used to treat TB in humans. Thus, individuals infected by *M. bovis* may present a treatment failure, which makes them potential transmitters of these resistant strains to other people and animals (Abrahão, 2005).

In industrialized countries, human infection with *M. bovis* has been largely controlled by pasteurization of cow's milk, inspection in slaughterhouses, and culling of cattle reacting to the compulsory diagnosis (Romero et al., 2006). In Brazil, despite the existence of a National Eradication Plan, clandestine meat and milk are marketed without sanitary control, which is a threat to public health; the ingestion of these products is a possible route of infection to humans (Abrahão, 2005). In some developing countries with uncontrolled bovine tuberculosis, most human cases occur in young persons, and result from drinking or handling contaminated milk (Cosivi, 1998).

Zoonotic TB can also be considered a socio-economic disease; it causes direct economic losses in agricultural areas and hampers the commercial exchange of animal products (Zumárraga et al., 1999). Many countries around the world support the control or eradication of bovine tuberculosis by national control programs, based on a test-and-slaughter policy. Brazilian policies regarding the control and eradication of bovine tuberculosis include the National Plan for Control and Eradication of Bovine Brucellosis and Tuberculosis (PNCEBT), written in 2001 and revised in 2004, which is based on the slaughtering of all animals reactive to the tuberculin tests. However, this traditional policy has not been fully successful in many countries, and new tools, including additional diagnostic tests and new vaccines, are urgently required (Pollock et al., 2005).

In cattle, tuberculous lesions are most often found in organs rich in reticuloendothelial tissue, particularly the lungs and associated lymph nodes, and the liver (Corner, 1990). Other studies conducted on naturally and experimentally infected cattle have demonstrated that the lesions are most commonly observed in the lower respiratory tract; however, the upper respiratory tract and associated tissues may also display disease in a significant number of cases. Although tubercles are not a pathognomonic lesion of cattle TB, the

presence of clinical signs of the disease is directly associated with their distribution and quantity (Medeiros, 2010).

Airborne infection is the most common transmission route, and more than 15% of cattle with BTB shed the mycobacteria, mainly early in the course of the infection (Cassidy et al., 1998). Studies with molecular markers have shown that infected cattle are a potential source of tuberculosis transmission to humans (Serrano-Moreno et al., 2008). Milk can be an important transmission route, resulting in extra-lung presentation of the illness (Wedlock et al., 2002). This was demonstrated in New York City, where 35 cases of infection by *M. bovis* were reported from 2001 to 2004; when they were traced back, some of the cases were associated with the consumption of cheese made with non-pasteurized milk, imported from an endemic area of BTB (CDC, 2005).

Using bacteriological culture methods, it has been calculated that only about 5% of tuberculin-reacting cattle (TRC) can eliminate *M. bovis* in milk. In such animals, the incidence of visible gross lesions in the mammary gland (MG) or supramammary lymph nodes (LN) is less than 0.5% (Goodchild and Clifton-Hadley, 2001). *M. bovis* has been isolated from milk samples from storage tanks, inadequately pasteurized milk, and milk samples from tuberculin non-reacting cattle (Pardo et al., 2001; Leite et al., 2003). This situation dramatizes the need for sensitive and accurate procedures for rapid identification of the bacteria in milk, to assist in the control of this zoonosis. PCR techniques offer high sensitivity, and have been successfully used for diagnosing BTB in several types of naturally infected organic materials such as tissue, blood and nasal exudates (Figueiredo et al., 2010; Cardoso et al., 2009; Romero et al., 1999). However, PCR techniques have been seldom tested for use in milk, particularly in Brazil (Zanini et al., 1998).

According to OIE, the proportion of zoonotic TB cases in Brazil is unknown (OIE, 2007), since bacteriological culturing for diagnosing TB is not employed routinely for all samples (Sequeira, 2005). The most recently published data estimating the incidence of zoonotic TB in Brazil (Kantor et al., 2008) reported the occurrence of only one occasion in 20 years (1987–2006), where *M. bovis* was confirmed as the etiological agent of human tuberculosis at the Hélio Fraga National Reference Laboratory (NRL) at the Federal University Hospital, Rio de Janeiro. In a two-year study, nearly 8,000 clinical samples were cultured for detection of mycobacteria, and no *M. bovis* isolate was obtained (Sobral, 2009). In the São Paulo laboratory network (Adolfo Lutz Institute), a total of 355,383 cultures were performed in the period 2001–2005, and only two *M. bovis* strains were recovered from two patients, one in 2001 from a lymph-node biopsy, and another in 2002 from a cerebrospinal-fluid sample. At the State Reference Laboratory in Rio Grande do Sul (1997–2005), of approximately 5,000 mycobacterial isolates phenotyped, no *M. bovis* was confirmed (Kantor et al., 2008).

Despite the presence of the disease in Brazil, there is a lack of official data concerning the current prevalence of bovine tuberculosis in the country. From 1989 to 1998, data from official reports indicate a national mean prevalence of 1.3% of infected cows (BRASIL, 2008). Since the implementation of PNCEBT in Brazil, the few studies reporting on the prevalence of the disease have provided estimates ranging from 0.7% to 3.3% (Baptista et al., 2004; Oliveira, 2007; Poletto et al., 2004; Ribeiro et al., 2003). According to the epidemiology of the disease, the higher incidence in dairy than in beef herds will also determine the geographical distribution. Roxo (2005) reported the rate of infection in different areas in

Brazil, and not surprisingly, the region with the lowest rate of infection is the one where beef herds are most predominant. Nevertheless, these data represent only particular regions, and cannot be used for estimates in the national context. It is important to keep in mind the enormous size of the Brazilian herd, which comprises approximately 200 million bovines (PAHO/WHO, 2006).

There is a growing perception that no single method is sufficient for detecting all cattle infected with BTB (Salfinger et al., 1994); therefore, a multidisciplinary approach must be employed, based on currently available methods. Some of the diagnostic methods and combinations of methods that are regularly used for diagnosing BTB are shown in Figure 1.

Bovine tuberculosis infection in cattle is usually diagnosed in the live animal. The diagnosis is based on delayed hypersensitivity reactions (intradermal tuberculin tests), a method that may lack both sensitivity and specificity. However, a definitive diagnosis is still established by isolation and identification of the etiological agent (*M. bovis*) from clinical samples, using a combination of traditional culture and biochemical methods, which is considered the “gold standard”. 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, but molecular methods, especially polymerase chain reaction (PCR) assays, are the most promising for diagnoses in live cattle (Serrano-Moreno et al., 2008; Figueiredo et al., 2010) and direct-detection *post mortem* diagnosis in bovine tissue samples (Cardoso et al., 2009; Liebana et al., 1995; Meickle et al., 2001; Romero et al., 1999; Vitale et al., 1998; Wards et al., 1995; Zanini et al., 1998; Zanini et al., 2001;).

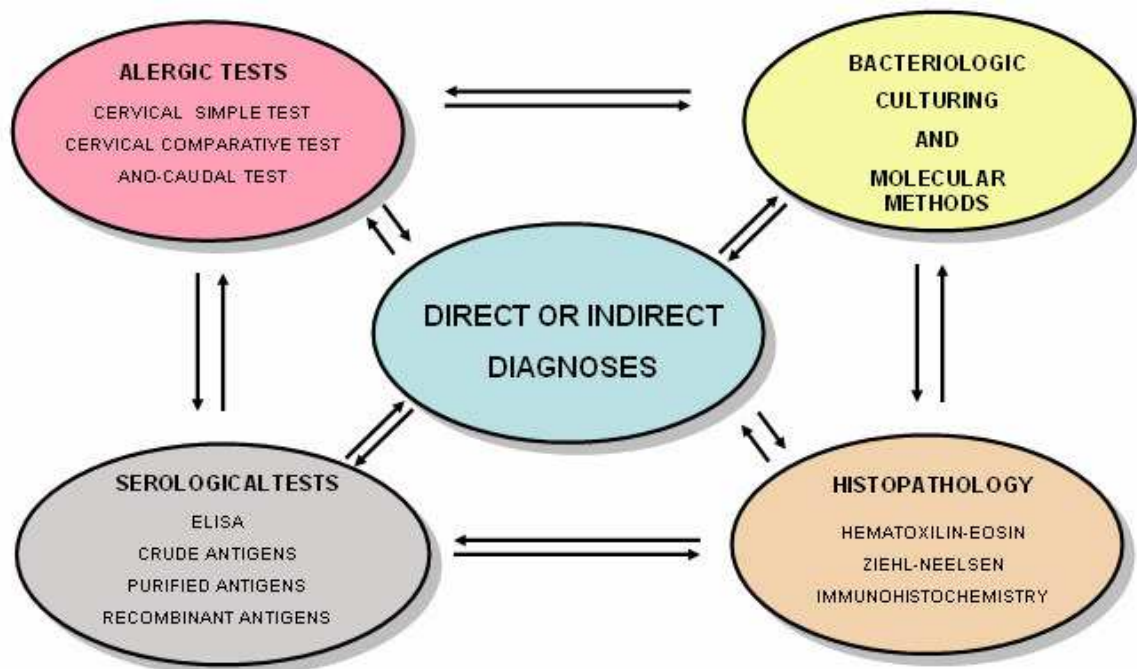


Fig. 1. Methods currently used to diagnose bovine tuberculosis (Medeiros et al., 2010).

The purpose of this chapter is to present new diagnostic approaches for the *Mycobacterium tuberculosis* complex in particular. We focus on discriminating *Mycobacterium bovis* by the use

of multiplex PCR and HPLC methods, which have been used to decrease the prevalence of this infection in countries where the disease still occurs.

2. Molecular methods in the diagnosis of bovine tuberculosis: Experiences from the field with a dairy herd in Brazil

Our studies were conducted on a dairy herd comprised of 270 adult crossbred Holstein and Gir cows, located in the Municipality of Macaé, state of Rio de Janeiro in southeast Brazil. Prior to the study, 21 adult cows had positive reactions to a Single Intradermal Tuberculin Test (SITT) and were kept in quarantine for 90 days, awaiting confirmatory tests. After 90 days, a Comparative Intradermal Tuberculin Test (CITT) was performed on these 21 cows (Group A), plus 29 selected cows that were negative for the first SITT test, including those with inconclusive results in the first test (Group B). A total of 34 animals reacted in the CITT (21/21 from Group A and 13/29 from Group B). From all 34 cows, milk samples and nasal swabs were collected and subjected to bacteriological culture, and the isolates were identified by the HPLC method and m-PCR, and also direct detection by m-PCR. All 34 cows were slaughtered 30 days after the injection of PPD, and thorough necropsies were performed. Mediastinal, scapular and retropharyngeal lymph nodes, as well as samples from the lungs were collected and also were analyzed by bacteriological tests, as well as HPLC and m-PCR.

2.1 Identification of *Mycobacterium bovis* isolates by a multiplex PCR (Figueiredo et al., 2009)

Several PCR systems have been developed for the detection of species belonging to the *M. tuberculosis* complex (MTC). The most commonly used system is based on primers that amplify segments of the IS6110 element, particularly targeting the 123-bp (Eisenach et al., 1990) and 245-bp fragments (Hermans et al., 1990). Another PCR system that has yielded successful identification of *M. bovis* isolates is focused on the amplification of a 500-bp DNA fragment in the RvD1Rv2031c genomic sequence (Rodríguez et al., 1999). A combination of conventional culture and biochemical techniques is the gold-standard method currently used for the identification of *M. bovis*, combining the isolation of the etiological agent and the unequivocal identification of the isolate. Mycobacteria were isolated from suggestive bovine tuberculous lesions, and the pure cultures of acid-fast bacilli (AFB) were identified by molecular analysis. The molecular assay consists of a single-step multiplex PCR (m-PCR), based on two set primers already tested and proved to be reliable, but not yet combined in a single PCR system. The combined PCR assay targets simultaneously the RvD1Rv2031c and IS6110 sequences, aiming to identify bacteria as MTC members as well as to distinguish *M. bovis* isolates from the other members of this complex.

Among the 50 adult cows from this herd that were tested by the intradermal tuberculin test according to official standards (Brasil, 2004), 34 animals were reactive, and were euthanized and necropsied. During the necropsy, a total of 91 samples of lymph nodes and lungs were collected, although not all the animals presented typical lesions. Samples were maintained under refrigeration, and tissues of each animal were processed together as one pooled sample per animal, totaling 34 samples. Samples were decontaminated using the Petroff method, inoculated on slopes of Lowenstein- Jensen medium with sodium pyruvate and

incubated for three months at 37°C. After growth, AFB-positive colonies were screened by m-PCR. Briefly, the mycobacterial DNA was extracted as described previously (Meickle et al., 20073). m-PCR was performed in a reaction mixture (50 µL) containing 5 µl of 10 × PCR buffer (Invitrogen®), 200 µM dNTP (GE Healthcare®), 2.5 U of recombinant *Taq* polymerase (Invitrogen®), 0.2 µM of each primer (Invitrogen®) JB21 (5'-TCGTCCGCTGATGCAAGTGC-3') and JB22 (5'-CGTCCGCTGACCTCAAGAAAG-3') (4) and INS1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS2 (5'-GCGTAGGCGTCCGTGACAAA-3') (10), 2.0 mM MgCl₂, and 5 µL of purified DNA template. Amplification was carried out in a GeneAmp PCR System 9600 (Applied Biosystems®) with the following cycling parameters: 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 1 min at 68°C and 1 min at 72°C, with a final extension at 72°C for 7 min. PCR products were checked by electrophoresis on 1.5% agarose gels stained with ethidium bromide (10 µg/mL). Negative samples were analyzed by PCR restriction analysis (PRA), using primers Tb11 (5'-ACCAACGATGGTGTGTCCA T-3') and Tb12 (5'-CTTGTCGAACCGCATACCCT-3') targeting for the *hsp65* gene (Telenti et al., 1993). The amplification products were digested with *Bst*E II and *Hae*III and the resulting fragments were fractionated by agarose gel electrophoresis and stained with ethidium bromide.

Mycobacteria colonies were isolated in Lowenstein-Jensen medium with sodium pyruvate from 17 of 34 (50%) processed samples, therefore confirming the infection. This herd had been TB-free in the last test, performed six months before the study. Therefore, we believe that the reactive cows had a recent infection, where visible lesions are not always present and the bacterial load is low. Considering the decontamination method used, it is not surprising that not all cultures yielded *M. bovis*. Nevertheless, it is noteworthy that the presence of some positive cultures is sufficient to characterize the outbreak of TB in this herd.

In these 17 isolates, m-PCR successfully amplified both target regions (the 500-bp fragment specific for *M. bovis* and the 245-bp fragment diagnostic for MTBC) in 15 of them (88.24%) (Figure 2, lanes 1-15). The two (11.76%) m-PCR-negative isolates (Figure 2, lanes 16 and 17) were confirmed by PCR-restriction analysis as *Mycobacterium* sp., but were not included in the *Mycobacterium tuberculosis* complex (results not shown).

PCR assays using primers JB21/JB22 have been considered to be highly reliable in identifying *M. bovis* isolates, showing 100% concordance with the conventional microbiological method (Rodríguez et al., 1999). However, the absolute specificity of JB21/JB22 primers for *M. bovis* has been disputed by another study, which reported that 13.3% (4/30) of *M. bovis* isolates failed to produce the 500-bp fragment (Sechi et al., 2000). Using specific primers for the IS6110 sequence, the 500-bp negative isolates were identified as belonging to the MTC, leading the authors to suggest that these isolates may lack the genomic target for JB21/JB22 primers. As this genotypic characteristic may not be infrequent, the use of a single primer pair can produce false negative results. On the other hand, an additional primer pair targeting for a different sequence, as in m-PCR, minimizes the occurrence of such false-negative results. The two sets of primers, although already described in the literature (Hermans et al., 1990; Rodríguez et al., 1999), as explained before, for the first time were combined to optimize a mPCR assay able to identify unequivocally *M. bovis* among mycobacterial isolates. The mPCR method was fast, reproducible and useful for the study of slow-growing mycobacteria, particularly in cultures where the small

number of bacilli hinders identification by classical methods. It also can be a valuable tool for the rapid identification of acid-fast bacilli isolated from suggestive bovine TB lesions.

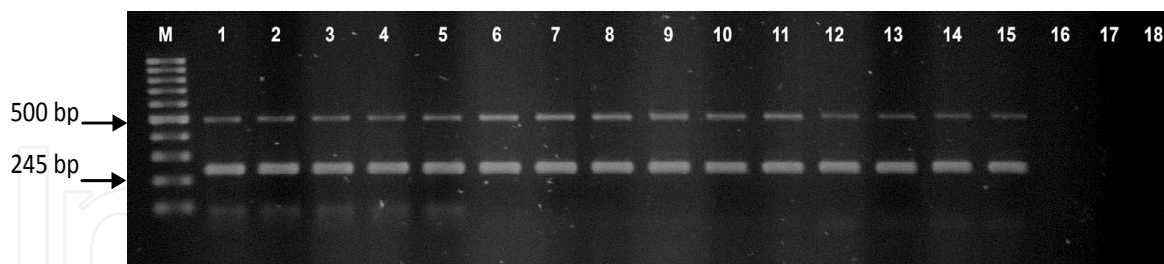


Fig. 2. **Identification of ABF isolates by m-PCR.** DNA extracted from 17 different acid-fast bacilli isolates was used as a template for m-PCR amplification of the *RvD1Rv2031c* and the *IS6110* sequences. Amplification products were separated by electrophoresis on 1.5% agarose gel and stained with ethidium bromide (10 $\mu\text{g}/\text{mL}$). Lane M: 100-bp DNA ladder (Fermentas®); lanes 1-17: m-PCR products of acid-fast bacilli isolated from suggestive BT lesions; lane 18: negative control. Arrows indicate the positions of the fragments of 500 bp (diagnostic for *M. bovis*) and 245 bp (diagnostic for MTBC members).

2.2 Detection of *Mycobacterium bovis* DNA in nasal swabs from tuberculous cattle by a multiplex PCR (Figueiredo et al., 2010)

The multiplex PCR-based method for the simultaneous detection of mycobacteria belonging to MTC and the specific identification of *M. bovis* was adapted to screen nasal swabs collected from live cows, suspected to be tuberculous. A total of 50 adult cows from a dairy herd with a previous history of bovine tuberculosis, including clinical cases, from Macaé were tested by the cervical comparative intradermal tuberculin test (ITT) with PPD (purified protein derivative) according to official standards (Brasil, 2004). 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 of cows examined) were slaughtered; the lungs and lymph nodes were removed and processed for bacteriology according to the OIE Terrestrial Manual (OIE, 2009). Briefly, after decontamination by the Petroff method, samples from lungs, 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 *M. bovis* and other mycobacteria belonging to MTC in nasal mucus was investigated by a single-step multiplex PCR (m-PCR) using two sets of primers, as previously described (Figueiredo et al., 2009), that targets simultaneously the *RvD1-Rv2031c* (specific for *M. bovis*) and *IS6110* (present in all MTC species) genomic sequences, but that to date had not yet been combined together in a single m-PCR assay. DNA was extracted from 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; 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) as described by Figueiredo et al. (2009). No mycobacterial growth was observed on agar slopes inoculated with nasal swab samples collected either from ITT-reactive or ITT-negative cows. On the other hand, mycobacterial colonies were observed in cultures from lung or lymph-node samples isolated from 17 of 34

PPD-positive cows (50% of the total of positive cows). In parallel, nasal swabs were examined 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 for use as templates in the m-PCR. The 500-bp fragment specific for *M. bovis* and the 245-bp fragment diagnostic for MTC were simultaneously observed in 2 of 34 (5.9%) of m-PCR reactions performed using nasal-swab DNA from ITT-reactive cows. Importantly, neither the 500-bp band nor the 245-bp band was found as m-PCR reaction products when swabs from ITT-negative cows were tested (results not shown).

M. bovis has been recovered from nasal exudates collected from cattle in naturally infected herds by using conventional culture techniques (de Kantor & Roswurm, 1978; McIlroy et al., 1986; Meickle et al., 2007). In these reports, recovery efficiencies varied from 8.7 (4) to 28.5%, when solely ITT-reactive animals were assessed (Meickle et al., 2007), regardless of the difficulty of the procedure, since it requires the presence of 10-100 viable organisms in the sample for a positive result, a condition attained only in advanced stages of the disease (Barry et al., 1993). Using PCR-based methods, the presence of species of the MTC group in nasal exudates of ITT-reactive animals was detected in 26% of the tested samples (Tejada et al., 2006), with some studies reporting detection rates of 50 or 58% (13,23), even though some PCR techniques may detect *M. bovis* using as little as 5 fg of DNA, which is equivalent to the amount of nucleic acid in a single mycobacterial genome (Estrada-Chávez et al., 2004).

The number of positive animals was smaller than expected, which was 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 (de la Rua-Domenech et al., 2006). None of the nasal-exudate samples from 34 ITT-reactive cows were found to be positive for the growth of *M. bovis*. Furthermore, 2 of 34 nasal-exudate samples (5.9%) were positive by m-PCR, a more sensitive and specific method than culturing (Meickle et al., 2007; Zanini et al., 1998; Zumárraga et al., 2005). These figures are lower than those previously obtained by using culture- or PCR-based methods to evaluate the presence of *M. bovis* in nasal exudates (de la Rua-Domenech et al., 2006; Meickle et al., 2007; Tejada et al., 2006; Vitale et al., 1998). The low rate of positive results may possibly be a consequence of the small numbers of viable bacteria present in nasal-swab samples, because the growth of the etiological agent was observed in cultures of lung and lymph-node samples from 17 of these cows, using the same procedure.

It has been well documented that in cattle experimentally infected with *M. bovis*, after each infection there is a lag period during which the etiological agent cannot be isolated from nasal mucus (Neill et al., 1998; McCorry et al., 2005; Kao et al., 2007). In a previously reported study, all experimentally infected animals shed *M. bovis* in the nasal mucus (Neill et al., 1998); but failure of some experimental animals to shed mycobacteria has also been reported (McCorry et al., 2005; Kao et al., 2007). Importantly, differences in the shedding profiles were observed, where those animals shedding *M. bovis* in nasal exudates were classified as either intermittent or as persistent shedders. It also appears that the overall level of shedding increases during the first four weeks after exposure and then begins to decline (Kao et al., 2007), although shedding can still be detected for many weeks, and in

some cases for several months. In conclusion, we have successfully used m-PCR assay to detect *M. bovis* in nasal exudates of naturally infected cattle, as previously reported (Meickle et al., 2007; Tejada et al., 2006; Vitale et al., 1998). 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 work better for the *in vivo* PCR-based detection of the microorganism than other fluids such as blood or milk. However, all these previous reports utilized primers to detect MTC species, and the identification of *M. bovis* was presumptive. The mPCR used here has the advantage of being specific for *M. bovis*, but 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, since shedding of mycobacteria in nasal mucus is required, the use of m-PCR for detecting live tuberculous animals by testing the nasal mucus could be 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 testing, thus assisting the bovine tuberculosis control and eradication program.

2.3 Detection of *Mycobacterium bovis* DNA in milk by m-PCR

Another valuable tool in confirming tuberculous cows is the identification of *M. bovis* in milk produced by the suspected animals. A PCR assay was developed for direct detection of *M. bovis* DNA in artificially and naturally contaminated milk. The assay used a pair of primers that were previously tested and proved reliable in targeting putative gene RvD1-Rv2031c.

Milk previously seeded with *M. bovis* was used as the starting material. The procedure involved DNA extraction by enzymatic lysing (proteinase K and lysozyme) and phenol:chloroform:isoamyl alcohol, followed by ethanol precipitation and m-PCR. The m-PCR was performed according to Figueiredo et al. (2010), and allowed us to detect *M. bovis* DNA in artificially contaminated milk, with a detection limit of 100 CFU/mL.

The use of the PCR method in spiked milk samples does not guarantee that it would perform equally effectively in the analysis of naturally infected samples. One could expect that in the latter, the interaction between the bacilli and the milk matrix could be more complex, and even that bacilli in milk might have already been killed by mammary macrophages (Zumarraga et al., 2005) and the DNA partially degraded. Therefore, the mPCR described here was evaluated for detection of *M. bovis* DNA in fresh unprocessed milk from CITT-reactive cows. A total of 50 adult cows from a dairy herd with a previous history of bovine tuberculosis, including clinical cases, from Macaé were tested by the cervical comparative intradermal tuberculin test (CITT) with PPD (purified protein derivative) according to official standards (Brasil, 2004). Thirty-four animals were CITT-reactive, and from all 50 cows, milk samples were collected (on the day that PPD was injected) and were subjected to bacteriological culture and m-PCR assay.

No mycobacterium growth was observed in CITT-negative cows (0/16). but in five milk samples collected from CITT-reactive cows (5/34) mycobacterial growth was observed. Only one isolate was confirmed as *M. bovis* by m-PCR (Figure 1, lane 1). Mycobacterial colonies were also observed in cultures from lung or lymph-node samples isolated from 17

of the 34 PPD-positive cows, and were confirmed as *M. bovis* by m-PCR (Figueiredo et al., 2009). In parallel, milk samples were tested for the presence of mycobacterium DNA using the m-PCR. The 500-bp fragment specific for *M. bovis* and the 245-bp fragment diagnostic for MTC were simultaneously observed in 4 of 34 (12%) m-PCR reactions performed with milk DNA templates from CITT-reactive cows (Figure 4). Importantly, neither the 500-bp nor the 245-bp amplicons were found when milk from CITT-negative cows was tested.

Similarly, in analyzing milk samples of cows from infected herds, previous studies have not detected any positive animal (Perez et al., 2002), while others targeting for the RvD1-Rv2031c, IS6110 sequence and MPB70 gene have reported amplifications from 2% to 28% of the cows (Romero et al., 1999; Zumárraga et al., 2005). Other studies using nested PCR (Serrano-Moreno et al., 2008; Vitale et al., 1998) also showed that the presence of *M. bovis* in milk is heterogeneous. The variable PCR results can be explained since the bacilli shed may be associated with cell-mediated immunity (CMI) in tuberculous cows (Pollock et al., 2001; Romero et al., 2006), as well as with epidemiological factors such as viral immunosuppression, metabolic imbalance, corticosteroids and peripartum (Doherty et al., 1995, 1996; Sordillo et al., 1997; Piccinini et al., 2006).

Thirty of the milk samples from CITT-reactive cows were negative by PCR. This suggests that some periods of bacterial excretion might have been missed, due to the design of the study, which included only one sampling. The intermittent character of bacilli secretion after a short constant post-infection period was documented by Menzies and Neill (2000). Another important point is that the 500-bp band was not found as a PCR product when milk from CITT-negative cows was tested, and mycobacterial colonies could not be isolated by culturing. The lack of recovery of *Mycobacterium* sp. could be due to the small number of excreted bacteria, or to the presence of dead or non-viable bacilli due to the action of macrophages, or even to the use of the Petroff decontamination method and reduced sensitivity of culturing compared with PCR (Zumárraga et al., 2005).

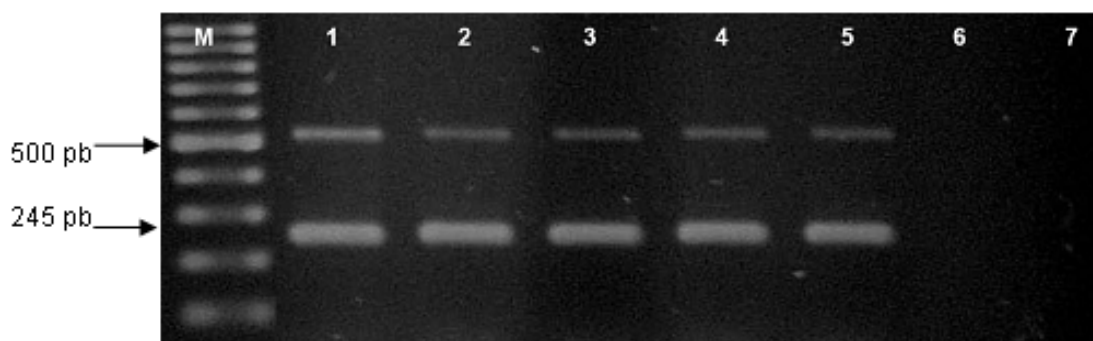


Fig. 3. Direct Detection of *M. bovis* DNA in milk samples from CITT-reactive cows. DNA templates obtained from 1 mL of milk samples were amplified by m-PCR of the RvD1Rv2031c and the IS6110 sequences. Amplicons were resolved on a 1.5% agarose gel stained with ethidium bromide. Lane M: 100-bp DNA ladder (Promega®); lane 1: positive control, *M. bovis* IP; lanes 2-5: milk samples from CITT-reactive cows; lane 6: negative control (water); lane 7: negative control (DNA template from *M. fortuitum* ATCC 6841). From each cow, three samples were analyzed and three independent experiments were performed.

The PCR assay allowed us to detect *M. bovis* DNA in artificially contaminated milk, with a detection limit of 100 CFU/mL, and also proved to be able to detect the bacilli in naturally infected milk. This method could be useful to assist the *in vivo* diagnosis for BTB, complementing the serological or microbiological tests, and is an alternative option in cases of mammary tuberculosis where the efficiency of serological diagnosis is nil. The method will be useful in epidemiological studies of BTB transmission and in quality control for the dairy industry, to prevent contaminated milk from entering the food supply.

2.4 Detection of *Mycobacterium bovis* DNA in bovine tissues by m-PCR

We adapted the m-PCR assay targeting the RvD1Rv2031c and IS6110 sequences, which are specific for *M. bovis* and MTC respectively, to identify *M. bovis* DNA in tissues from slaughtered positive-skin-test animals. The results are compared with those from the skin test and conventional culture for *M. bovis*.

Of 270 adult crossbred Holstein and Gir cows in a herd located in Macaé, 34 cows were considered CITT-reactive and also infected, by IFN assay (Marasi et al., 2010). At 30 days after CIIT, all 34 reactive animals were slaughtered and necropsied. Tissue samples were collected and analyzed by bacteriological methodology and m-PCR. DNA was extracted from lymph nodes, lung and udder tissues taken from the slaughtered animals, by a modification of a QIAamp Blood and Tissue Kit (Qiagen). One sample was selected per animal. A small piece of tissue (1-2 g) was macerated and an aliquot of 1 mL was taken. The 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 1 h at 37°C prior to 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 and eluted with 200 µL of the buffer.

The m-PCR was performed according to Figueiredo et al. (2010). In 17/34 (50%) samples *Mycobacterium* sp. isolates were obtained, and 15/17 were confirmed as *M. bovis* by m-PCR (Figueiredo et al., 2009). Direct m-PCR on tissue samples from CITT-reactive cows was positive for *M. bovis* DNA in 25/34 (73.5%) of the samples. All positive-culture specimens were also positive for m-PCR; and 10 (59%) samples that were negative by culturing yielded a positive result after m-PCR assay. It should be mentioned that the PCR was sensitive enough to detect *M. bovis* in a large proportion (59%) of those samples that failed to grow in culture, as also reported by Liebana et al. (1995), Zanini et al. (2001) and Araújo et al. (2005). The efficiency of the culture method used as a first criterion for *M. bovis* identification is low, because of the small number of live bacilli present in some tissues. Small numbers of live bacilli may be a consequence of a short delay in getting tissues to the laboratory, or may be due to the sensitivity of mycobacteria to the NaOH used in the Petroff method.

The improved identification shown here can be attributed to the removal of unwanted inhibitors. Ward et al. (1995) and Liebana et al. (1995) stated that "mycobacteria are difficult organisms from which to extract DNA and because they often exist as intracellular pathogens, may also be difficult organisms to purify from clinical samples, particularly tissues". Some compounds present in tissues, such as eukaryotic DNA or blood-originated inhibitory substances such as hemoglobin, lactoferrin and undegraded nucleic-acid samples from inflamed tissue can inhibit DNA amplification (Cardoso et al., 2009). On the other

hand, the use of the QIAamp Blood and Tissue Kit (Qiagen) circumvented those problems and supplied DNA templates suitable for amplification.

The nine remaining CITT-reactive cows were negative by both the culturing and m-PCR assays. Those results could be attributed to an inhibitory effect in the PCR assay (Al-Soud and Radstrom, 2001; Cardoso et al., 2009), and additional inquiries are needed with regard to DNA extraction methods. In addition, it should be considered that the tissue samples collected from those animals contained a low pathogen load, characterizing paucibacillary lesions that are commonly observed in recent infections occurring intra-herd. Two previous studies (Zanini et al., 2001; Cardoso et al., 2009) also reported a decreased efficiency in detecting mycobacteria in paucibacillary tissue samples. It is generally accepted that the CITT is a correlate of *M. bovis* infection and not necessarily of disease (Neill et al. 1994). In this study, CIIT-reactive animals developed disease, as demonstrated by the presence of lesions.

The results presented here indicate that m-PCR can detect *M. bovis* DNA in tissue samples, and represents a valid additional tool for the *post-mortem* diagnosis of BTB. Multiplex PCR is faster than culture-based detection, reducing diagnosis time from 120 to approximately 2 days, even when automated culturing with broth medium is used. Moreover, m-PCR is useful when the bacilli are non-viable and cannot be detected by culture methods. It can be of valuable help during sanitary inspection at slaughterhouses for condemnation of carcasses that show suspected lesions, or slaughtered animals suspected of having the disease. It is also important to note that a detailed inspection of bovine organs performed during necropsy in the field is more efficient than a rapid inspection at the slaughterhouse, because in the latter situation small lesions may be not detected.

2.5 Identification of species of the *Mycobacterium tuberculosis* complex by adapted High-Performance Liquid Chromatography (HPLC)

Complex high-molecular-weight β -hydroxyl fatty acids with a 22- or 24-carbon alkyl chain at the α -position are structural characteristics of mycolic acids, a fatty acid found in the *Mycobacterium* cell wall. By using several methods of fatty acid analysis, mycolic acids have been considered to be species- or group-specific (Butler et al., 1991). High-performance liquid chromatography (HPLC) analysis of mycolic acids has emerged as a reliable method for the diagnosis of mycobacteria, due to its rapid and reproducible nature, and because the mycolic-acid elution pattern observed for each mycobacterial species has generally been found to be unique, except for a few species that share the same pattern profile (Hagen & Thompson, 1995). The HPLC method has been considered a standard test for chemotaxonomic classification and rapid identification of *Mycobacterium* species by the Centers for Disease Control and Prevention (CDC) (<http://www.cdc.gov>), since 1990, and has been reported to achieve accuracy above 96% compared with DNA probe tests (Butler & Guthertz, 2001). A dedicated database, using adapted local protocols, must be developed in order to obtain chromatogram profiles from reference strains in the new analytic conditions, accrediting the local methodology and allowing for the correct analysis of clinical samples.

An HPLC method to identify *Mycobacterium* species, originally developed on a short column (CDC, 1996), was transferred to a longer column with similar stationary phase properties, but with a length of at most 33% of the initial one. Protocol modifications improved the

separation capabilities and the methodology specificity. Mycolic acids from 35 different reference *Mycobacterium* strains were saponified, extracted, derivatized, analyzed and successfully identified by the adapted HPLC method. The identification of mycobacteria was based on the relative retention times (RRT) of the chromatograms, comparing the profile obtained from the reference strains with profiles available in external databases. Although an internal standard was not used to align the chromatograms, the method showed good reproducibility and standardization, using the range of the relative standard deviation (RSD) of absolute retention time (ART) and the RRT, which varied from 0.68% to 0.97% and from 0.39% to 0.72%, respectively. The adapted method improved the identification of *Mycobacterium* species of clinical and veterinary interest, by comparing the new isolates with a database of mycolic acid chromatogram patterns from 35 reference mycobacteria strains, and comparing those profiles with those previously reported in the literature to enable identification of MTC species.

A suspension of acid-fast bacteria grown in LJ medium was removed with a swab and saponified with 2 ml KOH 25% in methanol:H₂O (v:v) autoclaved for 1 h at 121°C, 15 psi, to cleave the mycolic acids bound to the cell wall (Butler et al., 1991). Mycolic acids were then separated by acidification with HCl:H₂O (v:v) and extraction in chloroform. After conversion to ultraviolet (UV)-absorbing *p*-bromophenacyl esters (Pircen®) (Butler & Guthertz, 2001) and clearing with HCl:H₂O:Methanol (1:1:2, v:v:v), the mycolic acids were analyzed on a reverse-phase C18 100 × 4.6 mm column (Kromasil®) using high-performance liquid chromatography (Cage, 1994; Duffey et al., 1996). A gradient of methanol and dichloromethane (methylene chloride) generated by microprocessor-controlled pumps was used to separate the mycolic acid esters (Butler et al., 1991, Viader-Salvadó et al., 2007), which were detected with a UV detector at 260 nm (Du et al., 2008). Reproducible chromatographic patterns containing combinations of different diagnostic peaks (Butler et al., 1991, Glickman et al., 1994) were obtained by using reference strains (*M. abscessus* ATCC 19977, *M. africanum* ATCC 25420, *M. agri* ATCC 27406, *M. aichiense* ATCC 27280, *M. asiaticum* ATCC 25276, *M. aurum* ATCC 23366, *M. avium* ATCC 25291, *M. bovis* ATCC 19210, *M. bovis* BCG INCQS 00062, *M. chelonae* ATCC 35752, *M. flavescens* ATCC 14474, *M. fortuitum* ATCC 6841, *M. gastri* ATCC 15754, *M. godornae* ATCC 141470, *M. intracellulare* ATCC 13950, *M. malmoense* ATCC 29571, *M. mucogenicum* ATCC 49650, *M. scrofulaceum* ATCC 19981, *M. simiae* ATCC 25275, *M. terrae* ATCC 15755, *M. tuberculosis* ATCC 25177, *M. vaccae* ATCC15483 and *M. triviale* ATCC23292). Pattern recognition was done by visual comparison of the results for the reference strains with mycolic acid patterns from species of known mycobacteria (CDC, 1996, 1999). Identification of mycobacterial species by mycolic acid analysis was performed by visually comparing the UV patterns obtained from the samples with UV patterns from reference species, following recommendations of Butler and collaborators (Butler et al., 1991; Butler and Guthertz, 2001). Chromatographic patterns for each strain were examined for differences in the heights for pairs of peaks. HPLC patterns were grouped according to species, and the calculated values for each ratio were combined, sorted in numerical order, and examined for their ability to discriminate species, using the range of the relative standard deviation (RSD) of absolute retention time (ART) and the relative retention time (RRT). The 35 species were grouped into three general patterns (single, double and triple clusters) and divided accordingly into subgroups, according to Butler and Guthertz (2001).

A total of 21 *M. bovis* isolates from tissue, milk and nasal-swab samples from a dairy herd comprised of 270 adult crossbred Holstein and Gir cows, located in Macaé were confirmed by multiplex PCR (m-PCR) targeting for RvD1Rv2031c and IS6110 sequences, which are specific for the *M. bovis* and *M. tuberculosis* complexes, respectively (Figueiredo et al., 2009). Spoligotyping (Kamerbeek et al., 1997) was used to validate the HPLC methodology.

It has been reported that BCG-attenuated strains of *M. bovis* could be successfully differentiated from the MTC by HPLC (Floyd et al., 1992). This observation was confirmed in the present study, by comparing the chromatograms obtained from reference strains (Fig. 4). Other members of the complex, such as *M. bovis* and *M. tuberculosis*, were known to produce very similar chromatogram patterns, making it impossible to discriminate between them by this methodology. However, although requiring further work, the chromatogram profiles generated by the adapted elution protocol showed discrete and consistent differences in their chromatograms that could be used to discriminate them (Fig. 5-A). The simple and late-emerging single-cluster peak pattern group also included *M. asiaticum*, *M. gordonae* chromotype I (Fig. 5-B) and *M. kansasii* (Fig. 5-B). *M. triviale* was the only mycobacterium species present in this group, and it can be easily recognized (Fig. 5-B).

In these 21 isolates, m-PCR successfully amplified both target regions (the 500-bp fragment specific for *M. bovis* and the 245-bp fragment diagnostic for MTBC) in all isolates. A total of four spoligotypes were identified among the 21 *M. bovis* isolates. Two spoligotypes (SB0120 and SB0833) were described in the *M. bovis* spoligotype database (Brudey et al., 2006; www.mbovis.org). The other two represent novel, previously undescribed spoligotypes. The HPLC assay also identified the clinical *M. bovis* isolates as members of the *Mycobacterium tuberculosis* complex (Figure 6).

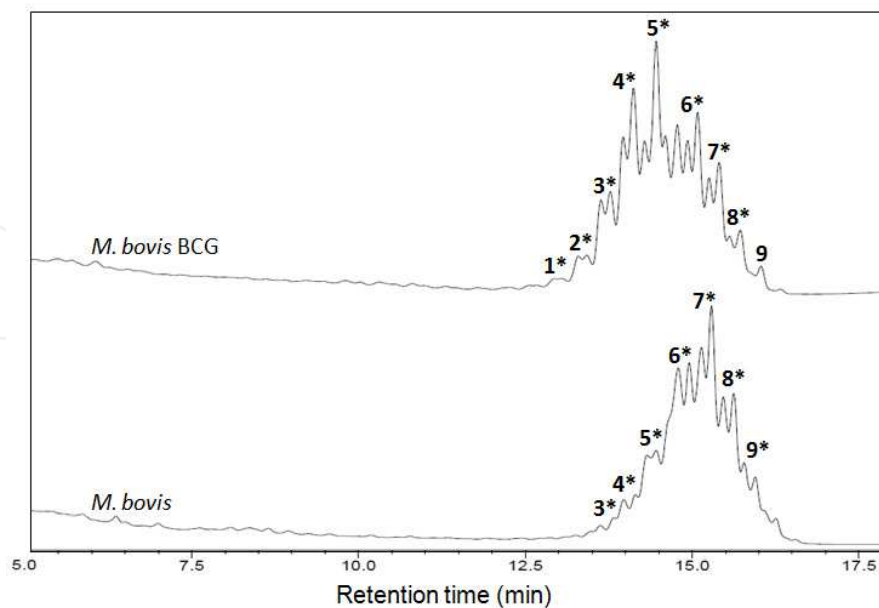


Fig. 4. Mycolic acid chromatograms from *M. bovis* BCG (INCQS0062) and *M. bovis* (ATCC 19210). * peaks showing a high degree of separation (appearing as a “double peak”), named according to Butler et al. (1991).

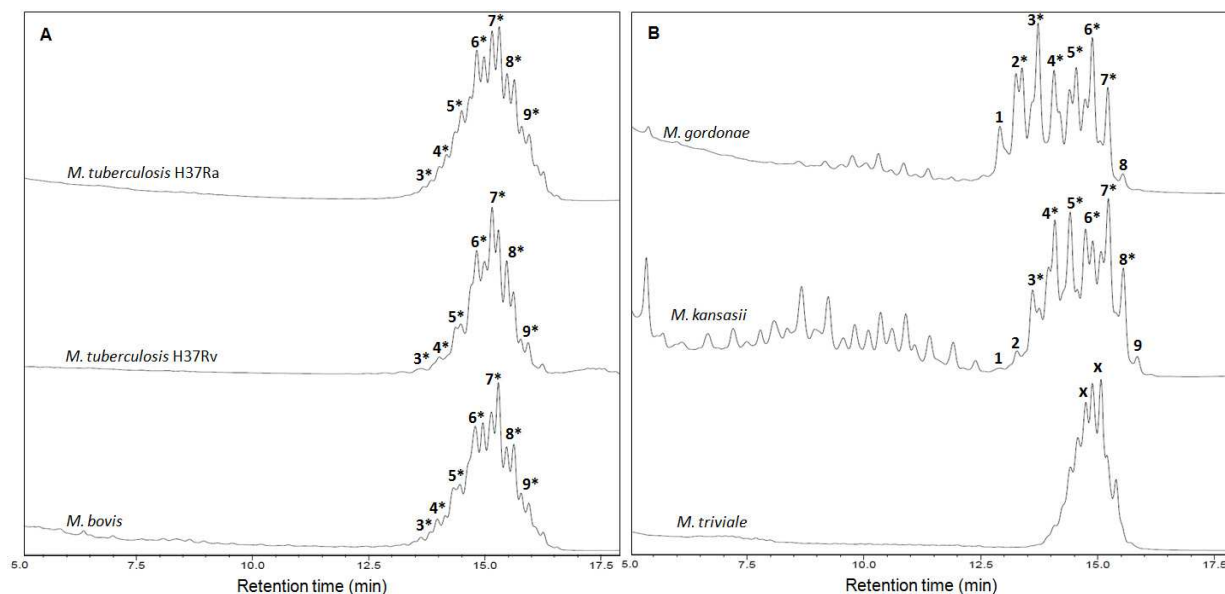


Fig. 5. Characteristic HPLC chromatograms of *Mycobacterium* species with late-emerging, simple, single-cluster peak patterns. **A)** *M. tuberculosis* H37Ra (ATCC 25177) and H37Rv (ATCC 27294) and *M. bovis* (ATCC 19210). **B)** *M. gordonae* chromotype I (ATCC 14470), *M. kansasii* (ATCC 12478) and *M. triviale* (ATCC 23292). *peaks showing a high degree of separation (appearing as a “double peak”), named according to Butler et al. (1991). *M. triviale* strain: xpeaks showing a high degree of separation (appearing as a “double peak”), compared to the chromatogram profile described by Butler & Guthertz (2001).

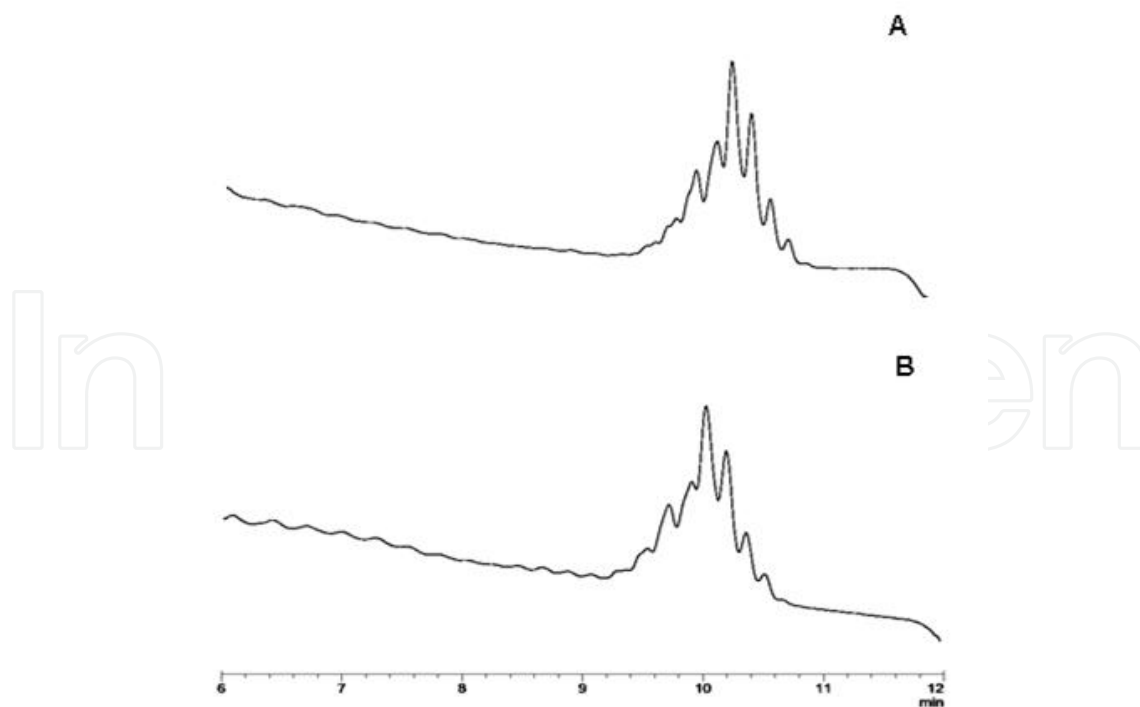


Fig. 6. Representative reverse-phase HPLC chromatograms of mycolic acid methylesters from reference strains and isolates: (A) *M. bovis* ATCC 19210; (B) 21 clinical *M. bovis* isolates from dairy herds in Brazil.

Identification of mycobacterium species using HPLC for mycolic acid analysis has proven to be rapid, reproducible and easily executed by several laboratories, making this approach one of the most appropriate methods to distinguish among the species. The separation capability using the modified method was superior to CDC patterns, and could be an alternative to allow discrimination between species with homologous HPLC chromatogram profiles.

3. Conclusion

Despite all efforts to control BTB, the disease persists, with serious implications for human health and the economy, particularly in the context of global trade. Because of the particular and complex characteristics of BTB, there is a growing realization that no single method by itself is sufficient to detect all the reactive animals in every stage of infection. Therefore, a multidisciplinary approach must be employed, using various categories of currently available methods. In a modern approach to the diagnosis and control of BTB, bacteriological, molecular, histopathological, and immunological assays must be employed, considering the indications, advantages, and disadvantages of each method. In this study we found that molecular diagnosis, combined with *ante mortem* and *post mortem* inspection, appeared to be a promising technique to improve the surveillance of BTB in herds, slaughterhouses, and the dairy industry, contributing to the success of the bovine tuberculosis eradication program.

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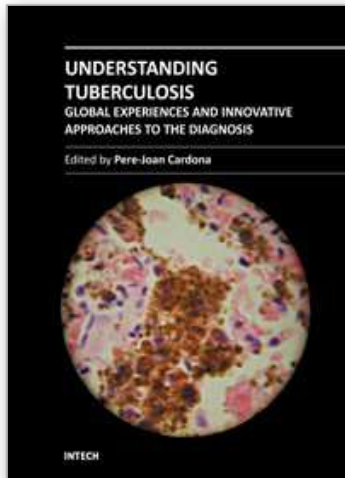
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Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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