Identification of *Mycobacterium bovis* among mycobacterial isolates from human clinical specimens at a university hospital in Rio de Janeiro, Brazil*, **

Identificação de *Mycobacterium bovis* em cepas micobacterianas isoladas de espécimes clínicos humanos em um complexo hospitalar na cidade do Rio de Janeiro

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Abstract

In 2005 and 2006, 8,121 clinical specimens submitted to the Mycobacteriology Laboratory of the Clementino Fraga Filho University Hospital/Thoracic Diseases Institute, in the city of Rio de Janeiro, Brazil, were inoculated on Löwenstein-Jensen medium containing glycerol and pyruvate. There were 79 mycobacteria isolates that presented growth only on pyruvate-containing medium, and those isolates were selected for the presumptive identification of *Mycobacterium bovis*. The selected isolates were screened with biochemical tests, PCR amplification (with the specific primers Rv0577 and Rv1510), and pyrazinamide susceptibility tests. All of the strains isolated showed specific phenotypical and genotypical patterns characteristic of *M. tuberculosis*, and no *M. bovis* strains were detected.

Keywords: Tuberculosis; Mycobacterium bovis; Polymerase chain reaction.

Resumo

Entre 2005 e 2006, 8.121 espécimes clínicos enviados ao Laboratório de Micobactérias do Hospital Universitário Clementino Fraga Filho/Instituto de Doenças do Tórax, no Rio de Janeiro, RJ, foram inoculados em meio Löwenstein-Jensen contendo glicerol e piruvato. Desses espécimes, 79 isolados de micobactérias tiveram crescimento somente em meio com piruvato, sendo selecionados para a identificação presuntiva de *Mycobacterium bovis*. Esses isolados foram submetidos à identificação por testes bioquímicos, amplificação por PCR com *primers* específicos (Rv0577 e Rv1510) e teste de suscetibilidade à pirazinamida. Todas as cepas apresentaram padrões fenotípicos e genotípicos de *M. tuberculosis*, não sendo detectado *M. bovis*.

Descritores: Tuberculose; Mycobacterium bovis; Reação em cadeia da polimerase.

Historically, *Mycobacterium bovis* has been associated with extrapulmonary tuberculosis in children, usually due to the consumption of raw milk from infected cows.[1] This framework is still in place in developing countries, especially in Africa, where there are reports that *M. bovis* has been isolated in 10-20% of children with cervical lymphadenitis.[2] In addition, in middle- and high-income countries, the pattern of *M. bovis* infection has changed, causing disease in hunters,[3] as well as in immunosuppressed individuals, such as alcoholics and HIV-infected patients.[4] In the United States, *M. bovis* infection has been identified in immigrant communities, particularly in those with a history of consuming unpasteurized dairy products.[5] Although bovine tuberculosis has been identified in Brazil,[6] there are no data available on human tuberculosis caused by *M. bovis* in the country. The lack of reporting might be due to the fact

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that few mycobacteriology laboratories routinely use culture media containing pyruvate. However, there is considerable epidemiological interest on the prevalence of zoonotic tuberculosis in Brazil. In addition, the fact that *M. bovis* is naturally resistant to pyrazinamide, one of the three drugs used in the treatment of tuberculosis, gives the isolation and identification of *M. bovis* in human clinical specimens a practical purpose in that this knowledge can inform decisions regarding treatment. The Mycobacteriology Laboratory of the *Hospital Universitário Clementino Fraga Filho|Instituto de Doenças do Tórax da Universidade Federal do Rio de Janeiro* (HUCFF/IDT-UFRJ, Clementino Fraga Filho University Hospital/Thoracic Diseases Institute, Federal University of Rio de Janeiro) receives approximately 5,500 samples annually. The hospital is a referral center for the diagnosis and treatment of severe forms of chronic and acute diseases, as well as of tuberculosis/HIV co-infection, in the state of Rio de Janeiro, Brazil. The objective of the present study was to investigate the prevalence of *M. bovis* in patients at the hospital.

Between 2005 and 2006, all clinical specimens referred to the Laboratory of Mycobacteriology of the HUCFF/IDT-UFRJ were decontaminated with the Kubica method,[7] and 0.2 mL were inoculated into two tubes of glycerol-containing Löwenstein-Jensen (LJ) culture medium and into one tube of pyruvate-containing LJ medium. The tubes were incubated at 37°C for up to 90 days.[7] The phenotypic identification of the *M. tuberculosis* complex was performed by means of biochemical tests (for niacin production, nitrate reduction, and thermal inactivation of catalase).[7,8] Specimens were also submitted to the pyrazinamide susceptibility test, as described by Canetti et al.[9]: culture medium (pH = 5.0-5.2) + 100 mg/mL of pyrazinamide. In that test, the critical proportion of pyrazinamide is 10%.[7] For the molecular identification, DNA extraction was carried out in accordance with national guidelines.[10] Amplification was performed according to Huard et al.[11] with modifications. In brief, two PCR mixes were prepared, each containing 1.25 mL of DMSO, 2.5 mL of 10× buffer, 0.75 mL of 50 mM magnesium chloride, 0.5 mL of 10 mM dNTP (A, T, C, and G), 13.8 mL of purified water, 0.2 mL of platinum Taq

polymerase (Invitrogen, Karlsruhe, Germany), and 5 mL of bacterial DNA, in a final volume of 25 mL. To each of the mixes, 0.5 mL of one of the primers used (Rv1510 and Rv0577) were added.[11] The PCR amplification was performed as follows: 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR product was run on a 1% agarose gel containing 0.5% ethidium bromide solution and observed under UV light.

During the study period, our laboratory received 10,861 clinical samples for the routine diagnosis of mycobacterial infection, of which 8,121 were inoculated into tubes containing LJ medium + sodium pyruvate, yielding 1,793 positive cultures. Of those, 79 mycobacteria strains recovered from the clinical specimens presented growth only on LJ medium containing sodium pyruvate, and those isolates were selected for the presumptive identification of *M. bovis*. The screening using three biochemical tests—niacin production, nitrate reduction, and catalase inactivation—showed that 57 isolates belonged to the *M. tuberculosis* complex, 14 were nontuberculous mycobacteria, and 8 strains did not grow in a second culture in a new tube with LJ (Table 1).

All *M. tuberculosis* complex isolates showed the standard *M. tuberculosis* biochemical pattern: niacin production, nitrate reduction, and negativity for catalase. In order to confirm these data, a few isolates were randomly selected and submitted to the pyrazinamide susceptibility test. Scorpio et al.[12] cloned the pyrazinamidase gene (*pncA*) from *M. tuberculosis* and *M. bovis* and found that a mutation was only observed in strains of *M. bovis* and of several subspecies of BCG. This mutation makes *M. bovis* and BCG intrinsically resistant to pyrazinamide.

Table 1 – Origins of the clinical specimens giving rise to isolates identified as belonging to the *Mycobacterium tuberculosis* complex or as nontuberculous mycobacteria.

Source of clinical specimen	*M. tuberculosis* complex	NTM
Pulmonary	45	14
Extrapulmonary	10	0
Unknown	2	0
Total	57	14

NTM: Nontuberculous mycobacteria.

Jong et al.[13] used the pyrazinamide test to identify *M. bovis* among 1,526 isolates and found that all 11 *M. bovis* isolates were resistant to pyrazinamide, compared with only 1.3% of the *M. tuberculosis* isolates. Of the 28 isolates identified as *M. tuberculosis* in the present study, only 3 were found to be resistant to pyrazinamide. In making the differential diagnosis between *M. tuberculosis* and *M. bovis*, it is advisable to use not only phenotypic tests but also genotypic markers,[14] and various molecular biology techniques have been used successfully in the characterization of *M. tuberculosis* isolates. Huard et al.[11] developed a PCR panel based on the deletions found in the complex in order to identify these species, using seven primers that amplified regions within the 16S rRNA loci. In the present study, we chose to perform PCR with two pairs of primers (Rv1510 and Rv0577) for better identification of *M. tuberculosis* isolates. The gene *Rv0577* is present in all species of the *M. tuberculosis* complex, albeit absent in most nontuberculous mycobacteria, whereas the *Rv1510* gene is known to be found in *M. tuberculosis, M. africanum* types I and II, and *M. canettii*, albeit absent in *M. bovis* and BCG. The 42 isolates that grew only on pyruvate and had a biochemical pattern consistent with *M. tuberculosis* were submitted to PCR, and all showed positive amplification for Rv1510, which is not found in *M. bovis* strains. Of the 42 isolates tested for Rv0577, 33 showed positive amplification, indicating that the isolates belonged to the *M. tuberculosis* complex (Table 2).

The results of the phenotypic and genotypic identification carried out in our study indicate that, during the study period, no *M. bovis* isolates were obtained. This result seems surprising because bovine tuberculosis is still prevalent in our country. In 2004, a study involving 454,108 animals showed a prevalence ranging from 0.37% in the southeast to 3.62% in the north.[6] In a study conducted in the state of Rio de Janeiro,[15] 12.7% of the dairy calves tested were found to be reactive to the skin prick test.

The present study was conducted at a tertiary hospital that serves as a referral center for transplant assessment, as well as for AIDS, cancer, and other diseases, which would facilitate the externalization of the *M. bovis* tuberculosis. Kantor et al.[16] extensively reviewed the international literature on zoonotic tuberculosis in Latin America, finding a prevalence ranging from 0.0% to 2.5%. According to the authors, human tuberculosis caused by *M. bovis* has been reported only in Argentina, Brazil, Ecuador, and Venezuela. The same authors showed that, during a 20-year period (1987-2006), the *Professor Hélio Fraga* Referral Center, located in the city of Rio de Janeiro and operating under the auspices of the Brazilian National Ministry of Health, identified only one case of tuberculosis caused by *M. bovis*, and that strain was isolated from the blood of an HIV-positive patient. From 2001 to 2005, the referral laboratories of the Adolfo Lutz Institute, located in the city of São Paulo, tested approximately 355,000 cultures using a pyruvate medium and isolated only two *M. bovis* strains, one from a lymph node sample and one from a liquor sample. In Rio Grande do Sul, a state with a tradition of animal husbandry, *M. bovis* was not identified among the 5,000 *M. tuberculosis* isolates tested. We were unable to identify any strain with an *M. bovis* profile among 1,793 mycobacteria isolates recovered during the study period.

One limitation of the present study is that the HUCFF/IDT-UFRJ primarily serves patients from urban areas. Although our data and those obtained by the other authors cited above showed no isolation of *M. bovis*, the presence of *M. bovis* in raw milk was demonstrated by two groups of authors in São Paulo.[17,18] Given that there is significant consumption of raw milk among the Brazilian population, these studies demonstrated that there is a potential risk of contamination by *M. bovis* that might lead

Table 2 – Results of additional tests for the identification of *Mycobacterium bovis* in the isolates tested.

Pyrazinamide susceptibility test result	PCR primer Rv0677		PCR primer Rv1510	Total
	Positive	Negative	Positive	
Susceptible	19	6	25	25
Resistant	2	1	3	3
Not performed	12	2	14	14
Total	33	9	42	42

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to infection and illness. However, there have been very few reports of tuberculosis caused by *M. bovis* in the country. Zoonotic tuberculosis in Brazil is definitely underreported. Cultures for mycobacteria are performed, in regional and national laboratories, only in special cases or for drug resistance surveys and only on glycerol-containing media, on which *M. bovis* grows poorly. It is also possible that the lack of isolation of *M. bovis* is the result of improved control activities of bovine tuberculosis. Supporting this hypothesis is the fact that, in 2001, the Brazilian National Program for the Control and Eradication of Brucellosis and Animal Tuberculosis made it mandatory to carry out skin prick tests in animals owned by milk production cooperatives.

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