Tuberculosis patients co-infected with *Mycobacterium bovis* in an urban area in Brazil

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The relative contribution of *Mycobacterium bovis* to the global tuberculosis (TB) is likely to be underestimated due to its dysgonic and slow growth characteristics and because of the absence of pyruvate in most used media is unfavorable for its primary isolation. In Brazil *Mycobacterium* culture, identification and susceptibility tests are performed only in TB referral centers, generally for particular cases. Furthermore, solid, egg-based, glycerol-containing (without pyruvate supplementation) Löwenstein-Jensen (LJ) or Ogawa media are routinely used, unfavouring *M. bovis* isolation. A cross-sectional study to determine the relative importance of *M. bovis* to the global tuberculosis in Juiz de Fora was performed. A total of 177 patients were culture positive for *Mycobacterium* spp. and also had the implied mycobacteria defined by conventional and/or molecular speciation methods. Among them, we detect only one *M. bovis* co-infection by conventional methods, in a pulmonary TB patient. But this supposed M. bovis profile couldn't be confirmed by molecular speciation methods. Also, supposed DNA present on formalin fixed and paraffin wax embedded biopsy tissue samples from TB patients were oxyR genotyped and 14 were found to be *M. tuberculosis* complex carriers. Two of them were recognized as M. bovis co-infections. Three (1.5%) were recognized as co-infections *M. bovis-M. tuberculosis* out 191 confirmed patients for *M. tuberculosis* spp. infections (95% CI = 0 - 3.3%). Our data indicate that *M. bovis* importance on the burden of human TB in Juiz de Fora, Minas Gerais, Brazil was low.

Key-words: zoonotic tuberculosis, oxyR pseudogene, Brazil

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INTRODUCTION

Although most cases of human tuberculosis (TB) are caused by *My-cobacterium tuberculosis*, concerns over *Mycobacterium bovis* have been expressed and are based on several observations. First, there have been outbreaks of multidrug-resistant (MDR) *M. bovis* strains among hospitalized patients with human im-munodeficiency virus (HIV) (*1*). These outbreaks highlight the high risk that MDR *M. bovis* could spread, especially in parts of Africa where animals with *M. bovis* and humans with HIV co-exist. Second, the reemergence of zoonotic tuberculosis among immigrants from regions where bovine tuberculosis is still prevalent have been documented (*2*).

In Latin America, the estimated proportion of zoonotic TB due to *M. bovis* accounts for 2% and 8% of pulmonary (PTB) and extrapulmonary (EPTB) forms, respectively (*3*), while in Brazil, the proportion of zoonotic cases due to *M. bovis* was estimated to be 3.5% of all TB cases in 1974 (*4*). As a standard procedure, sputum acid-fast bacilli (AFB) microscopy and histopathology are the major criteria for TB diagnosis in Brazil which may overlook potential cases of zoonotic TB cases in endemic areas of the country. This potential is reinforced as 0.85% of the cattle in Minas Gerais State (MG), Brazil, was demonstrated to be tuberculin reactors by the Brazilian Ministry of Agriculture (MABRAZIL).

The main objective of this cross-sectional study was to determine the proportion of *M. bovis* among patients who were attending two referral centers in Juiz de Fora County, a predominantly urban area of Minas Gerais State,

MATERIAL AND METHODS

Patients were recruited from two TB referral centers from March 2008 to February 2010. The first center (Center 1) is a TB regional hospital and the second center (Center 2) responds to local outpatient demands. Both centers treated 357 (75%) of all TB and mycobacteriosis cases reported in Juiz de Fora during the study period (n=476). Only patients with available clinical specimens or culture for mycobacteria characterization were included in this analysis (n=189). Most of the subjects who were excluded from the study were children and adolescents (<17 years old) or patients who had paucibacillary contact, in which situations the local physicians did not request direct microscopy for AFB or culture for mycobacteria on a routine basis. Written informed consent was obtained and the study was approved by the Ethical Research Review Board of the Federal University of Juiz de Fora (protocol no. 819.125.2006) and the Hospital Foundation of Minas Gerais State (protocol 52/08).

. Bacterial isolation was performed using only the conventional Löwenstein-Jensen (LJ) medium. The absence of pyruvate in conventional LJ could make the primary isolation of *M. bovis* more difficult. Therefore, changes in the procedures for the diagnosis of TB were implemented. All biological specimens underwent decontamination by Darzins' method, and the sediments that were obtained were inoculated simultaneously in LJ and Stonebrink (SB) media. Readings were taken until 60 days of incubation at 36.5°C . Biochemical tests (catalase at room temperature and at 68°C, niacin, nitrate, pyrazinamidase and urea) and the drug sensitivity testing (TSD) were performed to distinguish *M. bovis* from other *M. tuberculosis* complex-MTC (*5*). A portion of all positive AFB cultures was inactivated by heat (*6*) and DNA extraction (*6*) and molecular characterization were performed.

A two-step approach was used to distinguish *M. bovis* from other types of MTC. The first step was amplification of the *pncA* gene and detection of its polymorphism (*7*) by restriction enzyme analysis of the DNA from inactivated cultures (*8*). The second step was amplification of the partial sequence (150 bp) of the *oxyR* pseudogene (*9*) and detection of its polymorphism by restriction enzyme analysis of DNA from inactivated cultures (*10*).

Formalin-fixed and paraffin wax-embedded biopsy tissue samples from subjects with suspected EPTB (chronic granulomatous inflammatory process with caseous necrosis) were subjected to DNA extraction and amplification of *oxyR* psedogene (*11*) and detection of its polymorphism by restriction enzyme analysis (*10*) or DNA sequencing to determine which subjects were MTC carriers. DNA sequences were compared with those from GenBank.

Isolates with biochemical profiles of *Mycobacterium avium* complex (MAC) were confirmed by a PCR directed to carveol dehydrogenase (MAV_3017), which were designated by the MABRAZIL, following previous recommendations (*12*).

Descriptive analysis was carried out and the proportion of samples positive for *M.* bovis was estimated. Data entry and database management were performed using Epi Info software..

RESULTS AND DISCUSSION

A total of 189 tuberculosis patients had species profiles of mycobacteria that were characterized in an urban area in Brazil. There was a low prevalence of three *Mycobacterium bovis* co-infections and two *Mycobacterium avium* complex evidences among tuberculosis patients, which were being underestimated by local health services and deserve more attention.

All subjects with *M. bovis* infections in this study were co-infected with *M. tuberculosis*. In Mexico, a study also found a high proportion of *M. bovis* - *M. tuberculosis* co-infections in chronic cases of TB (*13*).

Among the three patients who had *M. bovis* co-infections, two had the *M. bovis* profiles confirmed by the *oxyR* pseudogene analysis from biopsies. DNA sequences from patients 1 and 2 showed a higher agreement compared to *Mycobacterium bovis* subsp. bovis AF2122/97 (GenBank no. BX248342.1) than *Mycobacterium tuberculosis* H37Rv (GenBank no. BX842579.1), and they presented adenine instead of guanine (Figure 1) at the position 285 of this pseudogene (*M. bovis* profiles).

Furthermore, an isolate from a pulmonary TB and AFB positive patient grew only in SB medium and had a biochemical profile of *M. bovis*, presenting a negative pyrazinamidase test result.

Two (11.7%) of the 17 extrapulmonary TB and one (0.6%) of the 170 pulmonary TB patients presented *M. bovis* profiles. Possible sources of *M. bovis* infection were unpasteurized cheeses, goats or slaughterhouse.

Furthermore, isolates of MAC were found respectively from sample of two patients. Nevertheless, as one of these cases were HIV/AIDS, it could be accepted that this were really co-infection cases.

Mtb: (...)CAGACGCTCGATGCTGCCCAACACCCGGGCCGGTGTGGCTGGCCACCG
MB: (...)CAGACGCTCGATGCTGCCCAACACCCGGGCCGCTGTAGCTGGCCACCG
P1 (...)CAGACGCTCGATGCTGCCCAACACCCGGGCCGCTGTAGCTGGCCACCG
P2 (...)CAGACGCTCGATGCTGCCCAACACCCGGGCCGGTGTAGCTGGCCACCG

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