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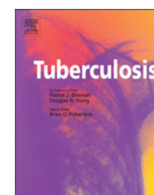

MOLECULAR ASPECTS

Genotyping did not evidence any contribution of *Mycobacterium bovis* to human tuberculosis in Brazil

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SUMMARY

The contribution of *Mycobacterium bovis* to the global burden of tuberculosis (TB) in man is likely to be underestimated due to its dysgonic growth characteristics and because of the absence of pyruvate in most used media is disadvantageous for its primary isolation. In Brazil *Mycobacterium* culture, identification and susceptibility tests are performed only in TB reference centers, usually for selected cases. Moreover, solid, egg-based, glycerol-containing (without pyruvate supplementation) Löwenstein-Jensen (L-J) or Ogawa media are routinely used, unfavouring *M. bovis* isolation. To determine the importance of *M. bovis* as a public health threat in Brazil we investigated 3046 suspected TB patients inoculating their clinical samples onto routine L-J and L-J pyruvate enriched media. A total of 1796 specimens were culture positive for *Mycobacterium* spp. and 702 TB cases were confirmed. Surprisingly we did not detect one single case of *M. bovis* in the resulting collection of 1674 isolates recovered from *M. bovis* favourable medium analyzed by conventional and molecular speciation methods. Also, bacillary DNA present on 454 sputum smears from 223 TB patients were *OxyR* genotyped and none was recognized as *M. bovis*. Our data indicate that *M. bovis* importance on the burden of human TB in Brazil is marginal.

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1. Introduction

Tuberculosis (TB) is primarily a disease of the lung, caused by very closely related mycobacteria belonging to the *Mycobacterium tuberculosis* complex (MTC). The complex includes the following species: *M. tuberculosis*, *Mycobacterium africanum*, *Mycobacterium bovis*, *and M. bovis-BCG, Mycobacterium microti, Mycobacterium caprae, Mycobacterium pinnipedii, and Mycobacterium canettii.*[1–3] By far, the most important agent of human tuberculosis is *M. tuberculosis*, accountable for more than 90% of cases.[4] Whereas *M. tuberculosis* is mainly a human pathogen, *M. bovis* has a broader host range, being mostly observed in cattle but sometimes reported as responsible for disease in other species. Noteworthy, *M. bovis* is naturally resistant to pyrazinamide, a first-line anti-TB drug.[5] Therefore, *M. bovis* detection, beyond its importance in the identification of outbreaks and epidemiological links, has also implications for planning the most appropriate anti-TB therapeutic regimen and public healthy interventions.

In the case of human TB, *M. bovis* is reported worldwide as being the second most frequent causative agent, estimated as being responsible for approximately 5% of the global tuberculosis cases.[6] Infection with *M. bovis* has accounted for a proportion ranging from 0.5 to 7.2% of all patients with a bacteriologically proven tuberculosis in industrialized countries.[7] However, it is generally though that in developing countries, *M. bovis* infection constitutes a more severe threat to public health and might account for up to 10–15% of the yearly registered new cases of human tuberculosis.[6–8]

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Human infection with *M. bovis* is mostly due to ingestion of unpasteurized milk or, alternatively, inhalation of droplet nuclei.[7,8] Although the anatomic site of human *M. bovis* disease is more often extra-pulmonary, human disease caused by *M. bovis* and *M. tuberculosis* are clinically, radiologically, and histopathologically indistinguishable.[9,10] Therefore, pathogen differentiation at species level requires the observation of phenotypic characteristics that include colony morphology, growth rate, and biochemical properties after culturing on appropriate medium.[10] In most developing countries, diagnosis of human TB is based on direct microscopic examination of specimens after acid-fast staining and chest radiography. Some TB reference laboratories perform culture on the *Mycobacterium* selective Löwenstein-Jensen (L-J) medium and biochemical tests to differentiate isolates, eventually including some that distinguish *M. tuberculosis* from *M. bovis*. However, even in these laboratories the use of the egg-medium containing pyruvate and semi-synthetic media in which the bovine bacillus would preferably grow is greatly limited.[11] Hence, the difficulty in obtaining primary isolates of *M. bovis* using conventional L-J medium is a major obstacle to ascribe the contribution of *M. bovis* to the burden of human TB in developing countries. To investigate the role of *M. bovis* in the etiology of human tuberculosis cases in Brazil, particularly in Rio de Janeiro, we conducted a study that added to conventional mycobacterial culturing and identification procedures, culturing on glycerol-free pyruvate containing L-J medium and genetic analysis. Additionally we performed direct genotyping of material present on Ziehl–Neelsen (Z–N) stained slides that had been prepared as part of standard TB diagnostic workout.

2. Materials and methods

2.1. Study setting and design

Virtually all commercially available milk supply is pasteurized in Brazil. Estimated Brazilian bovine population is 195 millions and the prevalence of tuberculin reactors animals range between 0.37% (in the Southern-Eastern) and 3.62% (in the Northern States).[12] Around 20% of Brazilian dairy cattle farms are thought to be affected by bovine tuberculosis. In a survey conducted in Rio de Janeiro state 12.7% of tested animals were tuberculin reactors.[13] However, the magnitude of bovine tuberculosis may be yet more serious in Brazil. Isolated animals and small herds, in areas comprising subsistence farming and pastoral communities, are usually not tested for *M. bovis* infection. Such areas are present even in the periphery of Brazilian metropolitan regions and people living in these areas may be exposed to unpasteurized milk and dairy products and meat of infected animals.

World Health Organization estimated as 92,000 the number of new human TB cases in Brazil for 2007 (an incidence rate of 48 per 100,000 individuals). The overall estimated prevalence of *M. tuberculosis* infection among adults was 60 per 100,000 inhabitants. The mortality rate from tuberculosis in Brazil was estimated to be 4.4 per 100,000 persons (8400 deaths/year in 2007).[14] Approximately 14% of adults with TB are thought to be HIV positive, and an estimated 20% of people living with HIV/AIDS have pulmonary tuberculosis.[15] Rio de Janeiro is the second largest city in Brazil, with 5.8 million inhabitants. In 2005, Rio de Janeiro City had 8713 TB cases and an incidence rate of 105.5 per 10^5 persons, almost twice as high as the rest of the country overall.[16] In total, 377 deaths related to TB were registered in 2003, with an incidence of 6.3 per 10^5 persons.[15] Official figures estimate that 8% of adult TB patients in Rio de Janeiro have HIV infection.[16] The Clementino *Fraga* Filho University Hospital/Institute of Thoracic Diseases of the Federal University of Rio de Janeiro (FURJ), serves a population of 1 million persons. Its Mycobacteriology Laboratory provides mycobacterial culture support to the University Hospital as well as to the City Health Care centers and performs 5000 cultures annually, 20% of which are positive for growth of mycobacteria. During a 20 months period (from April 2005 to November 2006) 8833 clinical specimens from suspected TB cases, routinely handled at our laboratory, were enrolled in the study. Additionally, to extend our investigation to regions where the risk for *M. bovis* infection is thought to be greater than in Rio Metropolitan area, samples from other two Brazilian regions and their related rural area (where pyruvate supplemented media and even L-J routine culture were not available) were also analyzed: 1) Campos, 300 km away from Rio de Janeiro, a mixed urban–rural municipality (0.5 million inhabitants) displaying unfavorable economic and human development indices that has its economy historically linked to the cattle farming, subsistence farming and sugar cane industry; 2) Four municipalities in the State of Minas Gerais having important cattle and dairy farming activities. Each geographical area represents a different eco-epidemiological setting. During a 12 months period (from December 2007 to November 2008) a total of 454 smear-positive samples corresponding to 223 pulmonary TB patients (some patients have more than one slide available to be examined) from these two regions were analyzed.

2.2. Handling of clinical specimens and culture conditions

As it is generally believed that *M. bovis* growth or, at least, optimal growth is constrained by using media without pyruvate supplementation and/or containing glycerol, we performed, in parallel to culturing on conventional L-J medium supplemented with glycerol, inoculation of all clinical specimens on tubes with L-J that was free of glycerol and supplemented with 5 mg/ml sodium pyruvate (Sigma–Aldrich, St. Louis, Missouri, USA). We collected a total of 8833 specimens from 3046 suspected TB patients and replicate patient samples were due either because of collection from different body sites and/or on different days. The median number of samples collected per patient was three. For culturing, clinical specimens were homogenized, submitted to *N*-acetyl-cysteine digestion and sodium hydroxide decontamination and centrifuged 2 times at 3000*g* for 15 min. Aseptically collected fluids and tissues were centrifuged without previous digestion or decontamination. The sediment was resuspended and divided into three equal volumes. Two 200 μl aliquots were plated onto L-J.[16] The third aliquot were plated onto L-J, without glycerol, pyruvate enriched agar slants (L-J pyruvate). All inoculated slants were incubated at 37 °C for eight weeks and submitted to standardized observation protocol, after 48–72 h, and later on twice weekly. After 60 days, bacterial counts were defined by colony numbering from 1 to 19, or scored +1 (20–100 colonies), +2 (>100 individual colonies) or +3 (confluent colonies).[17] Growth on the L-J slants resembling mycobacterial colonies (i.e., rough, tough and buff-colored), was subjected to Z–N staining and Gram staining to confirm the presence of acid-fast bacilli and rule out contamination. As a control procedure, we also inoculated four *M. bovis* (*M. bovis* ATCC 19210, *M. bovis* AN5, *M. bovis* [bovine clinical strain], *M. bovis* BCG 35736) and two *M. tuberculosis* strains (*M. tuberculosis* H37Ra ATCC 25177, *M. tuberculosis* [human clinical strain]) onto triplicate agar slants of both L-J media types (with glycerol or pyruvate) that were also incubated at 37 °C for 60 days. The mean of the triplicate was recorded as the colony count for the entire culture.

2.3. Phenotypic and biochemical characterization of bacterial isolates

For differentiation of *Mycobacterium* spp. of the MTC from mycobacteria other than tuberculosis (MOTT), we observed

characteristics based on pigmentation, colony morphology, speed of growth and biochemical tests.[17,18] Differentiation between some of the MTC members was achieved by analyzing phenotypical and biochemical features, including production of niacin, nitrate reduction, semi-quantitative catalase activity and catalase thermo stability at 68 °C, as previously described by Kent & Kubica.[17]

2.4. Genotyping

For genotyping of MTC DNA present in cultures a loop of bacterial mass was taken from cultures in L-J pyruvate medium, suspended in 0.5 ml of 10 mM Tris-HCl–1 mM EDTA–1% Triton X-100 and incubated at 100 °C for 10 min after vigorous mixing. Nucleic acids and processed cultures were stored at −20 °C until further use. *Spoligotyping* was performed using PCR and hybridization conditions on a commercially available membrane (Isogen Bioscience BV, Maarssen, The Netherlands) as previously described[19] and including (*M. tuberculosis* and *M. bovis* DNA) controls in every batch of tests. As a supplementary test for differentiation of *M. tuberculosis* from *M. bovis*, we performed *PCR-REA of a* 726 bp *fragment including the full pncA gene* (561 bp) as described by Barouni et al.[20] The *pncA* gene contains a polymorphic site at position 169 that permits the distinction between *M. bovis* and *M. tuberculosis.* In brief, the 726 bp fragment was generated using the primers 5′ GTC GGT CAT GTT CGC GAT CG 3′and 5′ GCT TTG CGG CGA GCG CTC CA 3′.[20] Thermal cycling conditions were: initial denaturation at 95 °C for 4 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 68 °C for 2 min and extension at 72 °C for 1 min. An additional extension step at 72 °C for 10 min was included at the end of 35-th cycle. PCR amplifications were digested with Eco065 I and resolved on a 5% agarose to determine the presence of the allelic variant of *M. Bovis*.

In addition to the genotyping of the clinical samples described above, we also performed genetic characterization of bacilli present in 454 Z–N stained sputum smears corresponding to 223 pulmonary TB cases. Patients were from two different Brazilian regions, including 140 (303 positive Z–N slides) from Campos, Rio de Janeiro State and 83 (151 positive Z–N slides) from four municipalities of the Minas Gerais State. The presence of acid-fast bacilli (AFB) was quantified by microscopy and recorded as + (less than 1 bacillus per 100 examined fields), ++ (1–10 bacilli per 50 examined fields) and +++ (more than 10 bacilli per 20 examined fields). The slides had been stored at room temperature for no longer than eight weeks before DNA extraction, the latter achieved by a procedure adapted from van der Zend et al.[21] Briefly, residual oil present on the glass slides was removed with xylol (Merck, Darmstadt, Germany), rinsed with distilled water and the biological material removed from the surface by scraping with a #14 lancet after spotting 25 μl of sterile distilled water. This suspension was transferred to a microcentrifuge tube containing 200 μl of a 15% Chelex 100 (Sigma–Aldrich, St. Louis, Missouri, USA) suspension and after vigorous shaking, incubated at 95 °C for 30 min. The samples were then centrifuged for 10 min at 13,000*g* and the supernatant removed and stored at −20 °C until further analysis. As single point mutations of the *pncA* gene[5] has been used to differentiate between *M. bovis* and *M. tuberculosis* PCRs were performed to amplify a 726 bp fragment including the full *pncA* gene. Almost all *pncA* PCRs failed to amplify. Assuming that the mycobacterial DNA present on the Z–N stained slides could have been significantly degraded, we realized that PCR amplification of targets of more than 150–200 bp may be difficult. For this reason we used primers previously described by Taylor et al. for amplifying a smaller 150 bp *oxy*R gene fragment[22] and determine nucleotide at *oxy*R gene position 285 to differentiate *M. bovis* from *M. tuberculosis.*[23] The sequence of these primers is 5′ CGCGCTGTCAGAGCTGACTTT 3′ (forward) and 5′ TCTGCGGAATCAGTGTCACC 3′ (reverse). The cycling conditions were: initial denaturation at 94 °C for 1 min; followed by 45 cycles consisting of 1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C. An additional extension step at 72 °C for 10 min was included at the end of 45-th cycle. After agarose gel electrophoresis, the 150 bp amplicon was purified and the presence of the single nucleotide A/G polymorphism at position 285, distinctive of *M. bovis*, was verified by sequencing. Sequencing reactions were performed using the BigDye Terminator 3.1 Cycle Sequencing kit and electrophoresis was performed under standard conditions on an ABI 3730 automated sequencer (Applied Biosystems). The sequence data were analyzed with SeqScape software version 2.5 (Applied Biosystems) and compared with the published sequence of oxyR (GenBank accession number U18263).

2.5. Data analysis

The two-proportion *Z*-test was used to compare mycobacterial growth on the different media used in the study. Fisher's exact test was used to compare the number of pulmonary and extra-pulmonary isolates that grew on L-J medium with those that grew exclusively L-J pyruvate medium. A significance level of 5% was considered. Spoligotype patterns were recorded both in a 43-digit binary format representing the 43 spacers and as an octal code[24] and initially verified for the presence of spacers that differentiate *M. tuberculosis* from *M. bovis*. In addition, *M. tuberculosis* patterns were introduced into a Microsoft Office Excel datasheet for cluster recognition and classification and then compared with the SpolDB4 database of the Pasteur Institute of Guadeloupe (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo) for type classification. All isolates that yielded spoligotypes that were recognized as not previously described patterns were submitted to at least one additional spoligotyping assay and only considered when confirming the first pattern.

3. Results

3.1. Mycobacterial growth on routine L-J and L-J pyruvate medium

3.1.1. Control strains

As someone could argue that inherent problems within the pyruvate supplemented medium might account for the failure of *M. bovis* "truly" positive specimens to yield identifiable mycobacterial growth we carried out a growth control assay. Standard collection and clinical MTC strains were inoculated on L-J and L-J pyruvate. Their growth rates on each and both media are shown in Table 1. All tested *M. bovis* reference strains grew adequately on pyruvate supplemented media.

3.1.2. Clinical specimens strains

From the 26,499 slants that were inoculated with 8833 clinical specimens from 3046 suspected TB cases 17,666 cultures on routine L-J and 8833 cultures on L-J supplemented with pyruvate, were observed during a 60 days period. Overall, 1796 clinical specimens out of 8833 (20.3%) produced 3365 positive mycobacterial cultures in one or both media. Most of specimens (1593, 88.6%) were pulmonary while 203 (11.4%) % were obtained from an extra-pulmonary site. The majority of the isolates (3001, 89.2%) were obtained from the 1593 pulmonary specimens. Among the pulmonary isolates, 66% originated from sputum, 27.1% from induced sputum, 6.9% from bronchoalveolar lavage fluid and 1% from other related specimens. An extra-pulmonary site was the origin of 364 (10.8%) isolates recovered from 203 samples. Taking into account the sum of extra-pulmonary isolates, 28% originated from lymph nodes, 27% from blood or bone marrow, 12% from pleura and the remaining 33% from various other tissues.

Table 2 shows the distribution of 3365 isolates recovered from 1796 culture positive specimens, obtained from 702 TB patients and

Table 1
Mycobacterium bovis and *Mycobacterium tuberculosis* collection and clinical strains culture yield in standard Löwenstein-Jensen (L-J) and glycerol-free Löwenstein-Jensen medium supplemented with sodium pyruvate (L-J pyruvate).

Mycobacterial strain	Culture Yield*	
	L-J medium	L-J pyruvate medium
Mycobacterium bovis ATCC 19210	5	+
Mycobacterium bovis BCG 35736	+	+
Mycobacterium tuberculosis H37Ra ATCC 25177	+	+
Mycobacterium bovis AN5	−	+
Mycobacterium tuberculosis human clinical strain	+	+
Mycobacterium bovis bovine clinical strain	−	+

* Mycobacterial culture yield expressed as null if no growth was observed after 60 days (−) defined by colony numbering from 1 to 19, or scored + (20–100 colonies). The mean of the triplicate was recorded as the colony count for the entire culture of each strain tested.

78 cases of MOTT infection (all enrolled at Rio de Janeiro), according to their medium or media of growth. Most of the pulmonary and extra-pulmonary strains grew on both L-J and L-J pyruvate medium: 1408 out of 1593 culture positive pulmonary samples (88.4%) and 161 out of 203 culture positive extra-pulmonary samples (79.3%). A similar proportion of pulmonary and extra-pulmonary strains were recovered from L-J pyruvate medium: 1478 from 1593 culture positive samples (92.7%) and 196 out of 203 culture positive samples (96.5%) (p-value = 0.062). Among the 1593 pulmonary specimens a comparable proportion of strains was recovered from L-J (1523/1593, 95.6%) and L-J pyruvate medium (1478/1593, 92.7%). However, for the 203 extra-pulmonary specimens the proportion of strains recovered from L-J pyruvate (196/203, 96.5%), was greater than that recovered from L-J medium (168/203, 82.8%) (p-value < 0.001). Some strains grew only on L-J or only on L-J pyruvate medium. Interestingly, when comparing the number of extra-pulmonary strains that grew exclusively on L-J (7/203, 3.4%) or exclusively on L-J pyruvate medium (35/203, 17.3%), extra-pulmonary strains appeared to express preferential growth on L-J pyruvate. Five-fold more extra-pulmonary mycobacterial isolation was observed exclusively on L-J pyruvate than exclusively on L-J medium (p-value < 0.001). Also, when comparing the number of pulmonary and extra-pulmonary strains that grew on L-J with those that grew exclusively L-J pyruvate (shown in Table 2), we observed a statistically significant higher number of extra-pulmonary strains recovery exclusively from L-J pyruvate medium (70/1523 *versus* 35/203, respectively for pulmonary and extra-pulmonary strains [$p < 0.001$]). No such difference was observed in the case of inoculates derived from pulmonary specimens.

3.2. Phenotypic and biochemical characterization of isolates

The great majority (1678, 93.5%) of the 1796 culture positive specimens resulted on the isolation of 3182 strains that were typed as MTC spp. while 118 (6.5%) resulted on the isolation of 183 MOTT strains. Most of MOTT isolates were recognized among pulmonary samples (108/118, 91.5%) and only 10 among extra-pulmonary ones (8.5%). Overall, considering the 3365 strains that grew on L-J pyruvate and/or L-J standard media, 183 (5.4%) were identified as MOTT and 3182 (94.6%) as MTC spp. It is noteworthy that inoculation on L-J pyruvate medium did not particularly favored growth or recovery of MOTT strains neither from pulmonary nor extra-pulmonary sites (data not shown). None of the 1674 mycobacterial isolates obtained from L-J pyruvate was identified as being *M. bovis.* All MTC isolates (from one or both media) presented values of phenotypic features and biochemical tests indicative of *M. tuberculosis*. Data concerning details about possible intermediate strains or grouping of isolates characteristics will be discussed elsewhere (manuscript *in prep.*). As it is commonly accepted that *M. bovis* growth or, at least, optimal growth is constrained by using media without pyruvate supplementation and/or containing glycerol, we performed the rest of the analytical procedures aimed at the identification of *M. bovis* as an etiologic agent of human TB only on isolates recovered from the L-J pyruvate, glycerol free medium.

3.3. Genotyping of bacterial isolates recovered from L-J pyruvate medium of patients from Rio de Janeiro

Among the 1674 isolates that were observed on L-J pyruvate 239 (14.2%) could not be submitted to genetic analysis, due medium degradation, contamination or loss during transfer and storage (153/239, 64%) or were excluded because they were identified as MOTT (86/239, 36%). The rest ($n = 1435$) was submitted to genotyping using the assays for *pncA* and spoligotyping. Workable PCR amplification and RFLP analysis of the *pncA* gene polymorphic site at position 169 was obtained for 1161 (81%) of the 1435 isolates and all showed the *M. tuberculosis* pattern. Good quality spoligo patterns were generated for 989 (69%) of the isolates and all presented genotypes characteristic for *M. tuberculosis* (using as reference data available on SpolDB4)[24] and SITVIT databases (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/). Data on genotyping outcome and source of the 1674 mycobacterial isolates recovered from L-J pyruvate medium are summarized in Table 3. The spoligotype results are provided as *M. tuberculosis* families and their frequencies among the analyzed isolates in Table 4. Considering SpolDB4 as a source for recognition of spoligotypes reported elsewhere, we observed that 858 of these isolates (86.8%) presented spoligotypes that were also present in the database while the remaining 131 (13.2%) segregated into 22 newly identified spoligotype patterns, not reported by SpolDB4. Among the formerly described genotypes, those belonging to the LAM family were most frequently observed (423/989, 42.7%), being mostly LAM9 (166/423, 39.2%) and about 70% (114/166) of these identified as LAM9-type 42. Other frequently observed families were H3 (117/989, 11.8%) with type 50 present in 78.6% of such isolates, T1 (117/989, 11.8%) with the type 53 being most frequent (45.3%), H1 (62/989, 6.2%) presenting type 47 in 84%

Table 2
Distribution of the 3365 isolates from 1796 culture-positive samples (corresponding to 702 TB cases) according to their growth in the Löwenstein-Jensen (L-J) and/or in the glycerol-free Löwenstein-Jensen medium supplemented with pyruvate (L-J pyruvate).

Source	N° Culture + specimens	Isolates growth pattern in L-J and L-J pyruvate medium				
		L-J	L-J Pyruvate	Both Media	Only L-J	Only L-J Pyruvate
Pulmonary	1593 (88.7%)	1523 (95.6%)	1478 (92.7%)	1408 (88.4%)	115 (7.2%)	70 (4.3%)
Extra-pulmonary	203 (11.3%)	168 (82.8%)	196 (96.5%)	161 (79.3%)	7 (3.4%)	35 (17.2%)
Totals	1796	1691 (94.2%)	1674 (93.2%)	1569 (87.3%)	122 (6.8%)	105 (5.8%)

The number of isolates is given (followed in parentheses by the percentage) in relation to the number of pulmonary (row 1), extra-pulmonary (row 2) and total (row 3) of culture positive specimens.
All 1796 culture positive specimens were obtained from 702 TB cases and 78 cases of MOTT infection enrolled at Rio de Janeiro.

Table 3
Summary data on genotyping outcome and source of the 1674 mycobacterial isolates from Löwenstein-Jensen pyruvate medium.

N° of Isolates *, †	Comment	Pulmonary	Extra-pulmonary
1674 (100%)	Total recovered from L-J pyruvate	1478 (88.3%)¶	196 (11.7%)¶
239 (14.2%)¶	Excluded**, not submitted to genotyping	213 (89.1%)	26 (10.9%)
1435 (85.8%)¶	Submitted to genotyping‡	1265 (88.1%)	170 (11.9%)
989 (69.1%)¶	Successfully spoligotyped	892 (90.2%)	97 (9.8%)
1161 (81%)¶	Successfully *pncA* genotyped	1042 (89.8%)	119 (10.2%)

* All 1674 Löwenstein-Jensen pyruvate isolates and 1691 standard Löwenstein-Jensen isolates (not shown in this table), from a total of 3365 isolates which were recovered from 1796 culture positive samples, corresponding to 702 TB patients and 78 cases of MOTT infection.
† All patients and samples were enrolled at Rio de Janeiro.
¶ The number of isolates is given (followed in parentheses by the percentage) in relation to the total number of L-J isolates (column 1, lines 2 and 3), to the total of 1435 isolates submitted to spoligotyping (column 1, lines 4 and 5) and to the number of isolates stated in the column 1 (columns 3 and 4).
** 153 isolates were excluded due medium degradation, contamination or loss during transport/storage and 86 because they were identified as MOTT.
‡ Spoligotyping and *pncA* genotyping were performed only on MTC isolates recovered from the L-J pyruvate medium.

of this grouping, LAM2 (58/989, 5.8%), LAM6 (56/989, 5.6%) mainly represented by type 64 (87%) and LAM3 (44/989, 4.4%).

3.4. Genotyping of MTC present on Ziehl–Neelsen stained smears

Mycobacterial DNA was obtained directly from AFB positive sputum smears produced during routine TB diagnosis workup in two different Brazilian regions (Campos, Rio de Janeiro State and four municipalities in the State of Minas Gerais) where cultures were not available. At first, we attempted to differentiate between *M. bovis* and *M. tuberculosis* as the mycobacterial species present on the sputum smears pointing out the distinctive *pncA* gene single nucleotide point mutation at position 169. However, in our hands almost all *pncA* PCRs failed to amplify directly from smears. The few *pncA* gene fragments (726 bp) that were successful amplified presented the *M. tuberculosis* pattern. Next, a smaller, 150 bp, oxyR gene fragment was amplifyed[22] and nucleotide at position 285 determined by sequence analysis to identify the species.[23] The *oxyR* genotyping fragment was successfully amplified and sequenced in 122 of the 303 (40%) AFB positive smears, corresponding to 49 of the 140 (35%) Campos TB patients and in 91 of the 151 (60%) AFB positive smears, corresponding to 58 of the 83 (69.8%) Minas Gerais TB patients. In all 213 successfully oxyR genotyped positive sputum smears, corresponding to 107 TB cases, the **G** nucleotide at position 285, characteristic of *M. tuberculosis,* was determined. Data on genotyping outcome of the 454 Z–N stained smears obtained from 223 pulmonary TB patients are summarized in Table 5.

Table 4
Mycobacterium tuberculosis spoligotype families and their frequencies among the 989 analyzed isolates.

Spoligotyping family	Number	Percentage
EAI5	4	0.4
EAI6_BGD1	1	0.1
H1	62	6.3
H2	2	0.2
H3	117	11.8
H4	1	0.1
LAM1	40	4.0
LAM1-LAM4	3	0.3
LAM2	58	5.9
LAM2-LAM4	3	0.3
LAM3	44	4.4
LAM3 and S/convergent	9	0.9
LAM4	17	1.7
LAM5	27	2.7
LAM6	56	5.7
LAM9	166	16.8
MAMU2	1	0.1
S	9	0.9
T1	117	11.8
T2	12	1.2
T2-T3	12	1.2
T3	22	2.2
T4	1	0.1
T4-CEU1	5	0.5
T5	1	0.1
T5_MAD2	6	0.6
U	34	3.4
U (likely H)	1	0.1
X1	3	0.3
X2	19	1.9
X3	5	0.5
New patterns	131	13.2
Total	989	100.0

4. Discussion

Human disease caused by *M. bovis* and other species of the MTC are similar, although the anatomic site of *M. bovis* disease is more often extra-pulmonary. Thus, collecting clinical samples from extra-pulmonary sites is an excellent strategy to identify cases of M. bovis human disease. Cases of human TB with *M. bovis* are mainly associated with the consumption of unpasteurized dairy products or close contact with infected animals.[7] Nevertheless, epidemiologic evidence supports the likelihood of human-to-human, airborne *M. bovis* contagion[25–28] and this issue has been considered as possibly relevant to the epidemiology and dynamics of human tuberculosis, at least in developing countries. Thus, studies aimed at the identification of the bovine bacillus in both, extra-pulmonary and respiratory samples, would be helpful to better understand the role of *M. bovis* as an etiologic agent of human TB. Results from a study comparing spoligo patterns indicate that the population of *M. bovis* strains causing human TB disease in Southern California is closely related to the *M. bovis* strain population found in Mexican cattle suggesting that human *M. bovis* disease in San Diego likely originated from Mexican cattle.[28] A recent report describes an outbreak of sputum-positive *M. bovis* disease transmitted by person-to-person contact in United Kingdom.[27] Moreover, Etchechoury et al. studying samples from 448 TB patients from different regions of Argentina, identified 400 isolates with representative PRA (PCR-restriction fragment analysis patterns of mycobacterium. Further analysis of samples with MTC profiles identified nine *M. bovis* strains indicating that that 2% of pulmonary tuberculosis was due to *M. bovis*.[29] In developing countries *M. bovis* infection is highly prevalent in farmed animals[13] and its contribution to the burden of human TB in these regions could be more important than it is generally thought. Also, it is well known that co-infection with HIV favors *M. bovis* transmission leading rapidly to disease development[30,31] and the high rates of HIV infection seen in most of the developing countries may increase the risk for the development human TB due to *M. bovis*. *M. bovis* grows poorly, or not at all, on standard glycerol-containing media but its growth is stimulated by sodium pyruvate. The pyruvate requirement of most *M. bovis* strains is the basis of one of the major phenotypic differences between *M. bovis* and *M. tuberculosis* and seems to be due to a point mutation in the pyruvate kinase gene, resulting in a defective glycerol metabolism.[32] Löwenstein-Jensen with pyruvate (but without glycerol), Stonebrink, modified Middlebrook 7H11 and tuberculosis bovine blood agar are considered the media of choice for the primary isolation of *M. bovis*.[33] We hypothesized that the extensive use of unfavourable media for *M. bovis* coupled with limited use of discriminatory biochemical and genotypic tests should be considered as a potential cause of under-reporting of *M. bovis*-related human disease. In

Table 5
Summary data and genotyping outcome of the 223 pulmonary TB patients and 454 diagnostic sputum smears used to perform direct *OxyR* genotyping from Ziehl–Neelsen stained slides.

	Geographical location		Totals
	Campos,* 300 km away from Rio	Four municipalities in the State of Minas Gerais†	
TB cases enrolled¶	140 (62.8%)	83 (37.2%)	223 (100%)
TB cases in which *oxyR* genotyping was obtained¶	49 (35%)	58 (70%)	107 (48%)
Smear-positive Samples studied¶	303 (67%)	151 (33%)	454 (100%)
Smear-positive samples successfully *oxyR* genotyped¶	122 (40.3%)	91 (60.3%)	213 (47%)
M. Tuberculosis oxyR genotype¶	122 (100%)	91 (100%)	213 (100%)
M. bovis oxy R genotype¶	0 (0%)	0 (0%)	0 (0%)

* Mixed urban–rural municipality, 0.5 million inhabitants.
† Teófilo Otoni (0.1 million inhabitants), Uberaba (0.3 million inhabitants), Montes Claros (0.4 million inhabitants), Juiz de Fora (0.5 million inhabitants).
¶ The number of patients and sputum smears are given (followed in parentheses by the percentage) in relation to the totals presented in column 4 (lines 1–4) and to the number of smears successfully *oxyR* genotyped (lines 4 and 5).

addition, the use of genotyping techniques to identify the bovine bacillus among MTC isolates that had grown on inappropriate, non-selective media may be contributing to the under-reporting of *M. bovis* human disease as well. To verify this hypothesis we investigated 3046 suspected TB patients plating their clinical specimens onto the routine L-J slants and onto one supplementary L-J pyruvate enriched, glycerol free, tube. Seven hundred two TB cases were confirmed. Mirroring the distribution of TB cases by site of disease in Brazil, 88% of the isolates originated from the respiratory tract. Most of pulmonary and extra-pulmonary isolates had grown well on L-J, L-J pyruvate or both media. However, some strains expressed preferential growth on L-J pyruvate medium, as reflected by five times more frequent mycobacterial isolation when comparing the number of extra-pulmonary strains that grew exclusively on L-J pyruvate medium (35/203, 17.3%) and exclusively on L-J (7/203, 3.4%) (p-value < 0.001). This trend of some extra-pulmonary strains to preferentially grow on L-J pyruvate medium could be due to: i) the higher proportion of extra-pulmonary isolates that, as most *M. bovis* strains, could also intrinsically present lesions in carbohydrate catabolism, namely glycerol kinase and/or pyruvate kinase null or defective activity; ii) pyruvate supplementation might favor bacterial recovery from the usually paucibacillary extra-pulmonary specimens enhancing growth performance of small inoculi even of *Mycobacterium* spp. not possessing intrinsic defects in carbohydrate metabolism.

Isolates of the MTC may be classified according to their phenotypic characteristics or biochemical properties as strains that completely fulfill the criteria for *M. tuberculosis* or *M. bovis*. For such strains, no further characterization is usually performed. Nevertheless, some strains do not match all the required diagnostic criteria and produce atypical phenotypic and biochemical identification test results. Sometimes the differentiation between these two close-related MTC species (>99.95% of genome sequence identity) by using classical methods may pose a challenge to the microbiologist and could generate misleading results.[34,35] Spoligotyping is considered as a reliable confirmatory genetic means for the differentiation between *M. bovis* and *M. tuberculosis* and it is particularly useful for strains with atypical biochemical identification test results.[35] For this reason, after the initial phenotypic and biochemical characterization, 1435 isolates recovered from the L-J glycerol free, pyruvate supplemented medium were submitted to spoligotyping. Patterns, adequate for analysis, were generated for 989 (69%) of these isolates. All of them were confirmed as MTC but none was marked by the distinct loss of spacers 3, 9, 16 and 39–43, individually or grouped, characteristic of *M. bovis*.[24] Actually, all successfully spoligotyped isolates were identified as *M. tuberculosis.* As samples were not selected for any particular purpose the 989 successfully spoligotyped strains and their spoligopatterns should be representative of the whole sample collection. However, it is possible that some of the strains not yielding spoligotyping were in fact *M. bovis*.

Using the spoligotyping technique, differentiation of MTC strains up to a subspecies level is also possible.[24] Therefore, to investigate the genetic diversity of *M. tuberculosis* strains circulating in Rio de Janeiro, we compared the obtained spoligopatterns with an updated international SpolDB4 database. Accordingly, the isolates were segregated in 33 strain families, the LAM (Latin-American and Mediterranean) spoligotype signature, characterized by the simultaneous absence of at least spacers 21–24 and 33–36, being the most frequently observed (423/989, 42.7%). Within this family, LAM9 was the most frequently identified family pattern (166/423, 39.2%). Other frequently observed families were H3, T1, and H1. The Beijing pattern was not identified among the strain families that could be grouped by SpolDB4. Overall, the proportion of strains distribution *M. tuberculosis* major clades and sub-clades observed here are in agreement with previous studies conducted in Rio de Janeiro and Brazil.[24,36,37] It is noteworthy that 13.2% (131/989) of the isolates segregated into 22 newly identified spoligotype patterns, not reported by SpolDB4.

Extending the search for human *M. bovis* TB cases to eco-epidemiological settings where the risk of *M. bovis* infection is thought to be greater than in Rio Metropolitan area, we also investigated samples from other two Brazilian regions and their rural areas harboring important dairy cattle activities and presenting high index of cattle *M. bovis* infection. As the L-J pyruvate supplemented media and even L-J solid media cultures are not available as a standard procedure during the TB diagnostic work in these municipalities of Rio de Janeiro and Minas Gerais States, we performed direct *OxyR* genotyping from 454 AFB positive Ziehl–Neelsen stained slides (303 from Campos and 151 from Minas Gerais, corresponding to 223 pulmonary TB patients) to identify any possibly present *M. bovis* strain. The *oxyR* 150 bp gene fragment was successfully amplified and sequenced from AFB-positive smears of 47.9% of the 223 studied pulmonary TB patients and in none, we observed the presence of the **A** nucleotide at position 285, typical for *M. bovis*.

It is somewhat surprising that not a single case of *M. bovis*-related TB was evidenced among the 1678 MTC culture positive samples from 702 TB patients, particularly considering that: i) a *M. bovis* favourable medium was systematically used in the study ii) the strains were isolated from 3046 suspected TB cases (702 confirmed) and a large number ($n = 8833$) of clinical specimens representative of a broad spectrum of tissues and sites, including lymph nodes, bone marrow, blood, bone and bowel biopsies; ii) the University Hospital being a regional and national reference for TB, AIDS, immuno-inflammatory diseases and hematological malignancies an important proportion of examined specimens were originated from immunosupressed patients and iii) the University Hospital Gastroenterology Clinic being a regional reference for cases of infectious, granulomatous and inflammatory bowel diseases several of such cases are routinely under diagnostic investigation that not rarely includes mycobacterial cultures. Also, as many of developing or

transition countries and regions, Brazil, and particularly Rio de Janeiro Metropolitan area and State, present high prevalence of both bovine and human TB and seems to present predisposing conditions for the development of human *M. bovis* infection and disease. Although almost all commercially available milk is pasteurized in Brazil it was recently reported that 11 of 128 (8.5%) bovine milk samples from retail markets in the State of São Paulo were contaminated by *M. bovis*.[38] In addition, small cow herds may be found scattered all over the country, even in peripheral regions of great cities like Rio. Small herd surveillance for infectious diseases presents problems in developed[39] and developing countries and is certainly much more difficult to be accomplished in countries like Brazil. Actually, in Brazil, for such dispersed cow or cows this surveillance may be completely non-existent. Moreover, a clearly defined slaughtering policy is not adopted in most Brazilian regions so people living in these areas are probably exposed to unpasteurized milk, unpasteurized dairy products and meat from infected animals. Even so, only few *M. bovis*-related TB cases have been reported in Brazil[11] and this picture remains unchanged even when lymph nodal and intestinal TB, TB associated with HIV and other immunosuppressive conditions and TB cases from Brazilian rural areas displaying poor economic indexes are taken in account. In a retrospective survey, Kantor et al. reported that the proportion of *M. bovis*-related human TB cases in 10 South America countries ranged from 0% to 2.5%.[11] Except for two cases from Ecuador, three from Brazil and one from Venezuela, all reported *M. bovis* TB cases were diagnosed in Argentina. Apparently in the other six investigated countries (Chile, Colombia, Costa Rica, Dominican Republic, Peru and Uruguay) *M. bovis* has never been isolated from humans.[11]

As vaccination with *M. bovis* BCG was introduced in Brazil in 1940 and has been systematically administered to virtually all infants since 1976, some particular degree of specific protection against the bovine tubercle bacillus provided by this thorough vaccination might be one possible explanation for the paucity of *M. bovis* TB cases reported in our country. Nevertheless, most expert comments on the burden of *M. bovis* in human TB emphasize that the problem is most probably underestimated due to the limited use of *M. bovis* favourable media and the inherent complexities of phenotypical and biochemical speciation within the MTC.[11] For this reason, our study design was optimized for culturing and identification of MTC species, using conventional and molecular tools, and the fact that we did not detect one single case of *M. bovis* in such a large sample collection indicates that *M. bovis* importance on the burden of human TB in Brazil is marginal.

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