Identification of *Mycobacterium bovis* in patients diagnosed with pulmonary and extrapulmonary tuberculosis

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Abstract

Introduction: In Mexico, there is an alarming increase in the number of cases of *Mycobacterium bovis* infection on pulmonary and extrapulmonary presentations. The lack of timely identification triggers complications and increases mortality. **Objective:** To know the frequency of *M. bovis* infections in clinical samples of patients with tuberculosis in the mycobacteria laboratory of a reference hospital in Mexico City. **Method:** Prospective, descriptive study. Strains isolated from biological material were studied in Löwestein-Jensen and MGIT1960 cultures. *M. bovis* was identified by amplifying the RD9 fragment with end-point polymerase chain reaction (PCR). **Results:** Eight-hundred and fifty tuberculosis-diagnosed patients were included; in 441 cases, *Mycobacterium tuberculosis* was confirmed by positive culture (250 pulmonary, 65 ganglionic, 39 renal, 34 meningeal, 25 milary, 14 pleural, 8 peritoneal, 4 bone and 2 pericardial cases). Forty-eight strains (10.8%) were typified as *M. bovis* by amplification of the RD9 fragment with end-point PCR. **Conclusions:** *M. bovis* is not currently thought of a causative agent of tuberculosis, which could be the cause of pharmacological treatment failure. In this study, the main extrapulmonary form was observed to be cervical lymphadenopathy.

KEY WORDS: Tuberculosis. *Mycobacterium bovis*. End-point polymerase chain reaction.

Identificación de *Mycobacterium bovis* en pacientes con diagnóstico de tuberculosis pulmonar y extrapulmonar

Resumen

Introducción: En México existe un incremento alarmante de casos de infección pulmonar y extrapulmonar por *Mycobacterium bovis*. La falta de identificación oportuna deriva en complicaciones y eleva la mortalidad. **Objetivo:** Conocer la frecuencia de infecciones por *Mycobacterium bovis* en muestras clínicas de pacientes con tuberculosis, identificadas en el laboratorio de micobacterias en un hospital de concentración de la Ciudad de México. **Método:** Estudio prospectivo, descriptivo. Se estudiaron cepas aisladas de material biológico en cultivos Löwestein-Jensen y MGIT1960. La identificación de *Mycobacterium bovis* se realizó mediante la amplificación del fragmento RD9 por PCR punto final. **Resultados:** Se incluyeron 850 pacientes con diagnóstico de tuberculosis, en 441 casos se confirmó *Mycobacterium tuberculosis* por cultivo positivo (250 casos pulmonares, 65 ganglionares, 39 renales, 34 meníngeos, 25 miliares, 14 pleurales, ocho peritoneales, cuatro óseos y dos pericárdicos). Se tipificaron 48 cepas (10.8 %) como *Mycobacterium bovis* por amplificación del fragmento RD9 por PCR punto final. **Conclusiones:** Actualmente no se piensa en *Mycobacterium bovis* como agente causal de tuberculosis, lo que pudiera ser la causa del fracaso del tratamiento farmacológico. En este estudio se observó que la principal forma extrapulmonar es la linfadenopatía cervical.

PALABRAS CLAVE: Tuberculosis. *Mycobacterium bovis*. PCR punto final.

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Introduction

Mycobacterium tuberculosis is the main causative agent of tuberculosis in humans, whereas *Mycobacterium bovis* normally causes tuberculosis in animals.¹ The term zoonotic tuberculosis is used to describe *Mycobacterium bovis* infection in humans.

Tuberculosis transmission caused by both pathogens can occur from humans to animals and vice versa.² The World Health Organization points out that, currently, it is a public health problem that is poorly addressed in emerging countries. In recent studies, *Mycobacterium bovis* and *Mycobacterium tuberculosis* coinfection has been observed.³

The infection is acquired by direct and indirect contact with animals and their secretions, which increases the risk of zoonotic tuberculosis. Known risk factors include working history of the individual, consumption of raw milk and raw dairy products, especially in extrapulmonary tuberculosis cases.^{3,4}

Mycobacterium bovis genotyping has been mainly carried out based on molecular patterns (spoligo-types), which are highly useful to know the spread of the disease; unfortunately, it is an expensive study and it is not available in all laboratories of the country.⁵

In Mexico, the control of this disease is carried out by the National Program against Bovine Tuberculosis, which has managed to reduce the prevalence of the disease in most part of the country, except in regions of high milk production.⁶

Lung infections and extrapulmonary presentations are not adequately documented or recorded; they are believed to be responsible for 5 to 10 % of all tuberculosis infections. Not all *Mycobacterium bovis*-infected individuals develop active disease, although patients with immunosuppression are more prone to infection.^{5,6}

One of the challenges health personnel is faced with lies in identifying the cause of the disease, given that *Mycobacterium bovis* and *Mycobacterium tuberculosis* are clinically and radiologically indistinguishable from each other. The first case is suspected when a patient does not improve with first line treatment, which makes it essential for the species involved to be known in order to establish a specific treatment, since *Mycobacterium bovis* is naturally resistant to pyrazinamide.⁷

The purpose of this study is to determine the frequency of *Mycobacterium bovis* in clinical samples of patients with pulmonary and extrapulmonary tuberculosis, processed in the mycobacterial laboratory of a

tertiary care specialty hospital, from January 2013 to December 2018.

Method

A prospective study was designed, where clinical samples of patients admitted with a diagnosis of tuberculosis between January 2013 and December 2018 were used to establish the frequency of *Mycobacterium bovis* as etiological agent in subjects with pulmonary and extrapulmonary tuberculosis forms at General Hospital of Mexico "Dr. Eduardo Liceaga". Inclusion criteria were: patients older than 18 years in whose clinical samples (sputum, biopsy material, urine, pleural fluid and cerebrospinal fluid) mycobacterial growth was obtained in the Löwestein-Jensen and MGIT960 culture medium, without previous tuberculosis diagnosis or anti-tuberculosis drug treatment.

At hospital admission, every patient diagnosed with tuberculosis underwent history taking, blood cytometry, blood chemistry, human immunodeficiency virus identification by ELISA with confirmation thereof by Western-blot, and chest X-ray.

In extrapulmonary presentations, biopsy was performed (cervical lymphadenopathy, peritoneum, pleura and bone tissue). The obtained samples were fixed in 10 % formalin and embedded in paraffin, sectioned and stained with hematoxylin-eosin for microscopic examination. The tuberculosis diagnosis was established when the findings included granuloma formation with caseous necrosis foci, fibrosis and presence of epithelioid cells.

The biopsy material was taken with aseptic technique and subjected to culture for mycobacteria with Löwestein-Jensen and MGIT960 medium, the gold standard for tuberculosis diagnosis.

Each sample underwent Ziehl-Neelsen staining for AARB, adenosine deaminase (ADA), endpoint polymerase chain reaction (PCR) for the detection of the 1S6110 insertion sequence present in mycobacteria of the *Mycobacterium tuberculosis* complex, and a skin test with purified protein derivative (PPD-RT23, Statens Seruminstitut, Copenhagen, Denmark) was carried out.

The specimens were homogenized in isotonic saline, buffer phosphate and decontaminated with a 4 % sodium hydroxide solution. After neutralization and centrifugation, each homogenate was re-suspended in distilled water. Acid-alcohol resistant bacilli (AARB) were detected with Ziehl-Neelsen staining. For the cultures, 0.2 mL of sample homogenates were

inoculated in bottles with MGIT 960 fluorescent broth (Mycobacterium Growth Indicator Tube, Becton Dickinson, San Jose, CA, USA) and Löwestein-Jensen solid medium. The cultures were incubated at 37 °C for eight weeks and inspected weekly to assess for growth.

DNA was extracted by thermal shock from a bacterial suspension of each isolate and was used for amplification of approximately 124 bp of the product corresponding to an insertion sequence, IS6110, which is characteristic of the *Mycobacterium tuberculosis* complex. PCR primers IS6110F (5'-GATCCGCCAGC-CCA-GGATCCTGCG-3') and IS6110R (5'-AGGTGCG-GACCACCAGCACCTAACC-3') were designed at the Institute of Epidemiological Diagnosis and Reference. The PCR mixture (50 µL) was 10 mM Tris-HCl, 1.5 mM magnesium chloride, 50 mM potassium chloride (pH 8.3), 1 U taqpolimerase, 2 mM of a dNTP mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.2 µM L of DNA from each primer. The reaction conditions were as follows: one 1-minute cycle at 94 °C, followed by 45 15-second cycles at 94 °C, 50 seconds at 70 °C and one 7-minute cycle at 72 °C.

For *Mycobacterium bovis* molecular identification, the DNA preparation was carried out using the boiling method, and it was used as a template for generic PCR of the *Mycobacterium* complex. To differentiate between *Mycobacterium tuberculosis* and *Mycobacterium bovis*, all strains reported as positive for the mycobacteria complex by PCR were analyzed with an RD9 end-point PCR system with three primers (RD9F, RD9R and RD9I), using the zs|2ew method by Samir Das.⁸

To confirm and differentiate *Mycobacterium bovis* from *Mycobacterium tuberculosis*, a real-time PCR assay was developed, based on the presence or absence of differentiating regions (RD1, Ext-RD9, RD4, RD9 and RD12) between the genomes of the MTBC members.

Results

During the study period, 850 patients with suspected tuberculosis were admitted. In 441 cases, *Mycobacterium tuberculosis* was confirmed by Löwestein-Jensen and MGIT960 positive culture, with an average strain growth of 37 days. Twelve cultures were eliminated due to contamination; 70 % (310) of patients were males and 30 % (131), females; mean age was 41 years. PPD was positive with induration larger than 10 mm in 25 % of patients.

Figure 1. *Mycobacterium tuberculosis/Mycobacterium bovis* differentiation by end-point PCR. Analysis of the RD9 fragment in 2.5 % agarose gel. MPM, molecular size marker VIII (ROCHE); 1, *Mycobacterium tuberculosis* H37Rv strain; 2, PCR reagents control; 3, *Mycobacterium bovis* BCG; 4 and 5, samples 3209 and 1687, respectively, identified as *Mycobacterium tuberculosis*; 6, sample 1916 identified as *Mycobacterium bovis*.

In 30 % of cases, acquired immunodeficiency syndrome (HIV/AIDS) was identified, in 25 %, diabetes mellitus, and in 7 %, a history of neoplasms on treatment with chemotherapy; there were five patients with chronic use of corticosteroids.

Predominant symptoms were fever (85 %), cough (82 %), weight loss (78 %), asthenia and adynamia (72 %), dyspnea (60 %) and hemoptysis (20 %).

Of the 441 cases with positive cultures, 250 (56.6 %) had pulmonary tuberculosis; 65 (14.7 %), lymph node tuberculosis; 39 (8.8 %), renal tuberculosis; 34 (7.7 %), meningeal tuberculosis; 25 (5.6 %), miliary tuberculosis; 14 (3.1 %), pleural tuberculosis; eight (1.8 %), peritoneal tuberculosis; four (0.9 %), bone tuberculosis; and two, (0.4 %) pericardial tuberculosis. In all tuberculosis-diagnosed cases, treatment with isoniazid, rifampicin, pyrazinamide and ethambutol was started, which was strictly supervised. Clinical response was evaluated for one year after diagnosis. In the mycobacteria typing, *Mycobacterium tuberculosis* was identified in 89.2 %.

In the present study, 48 strains (10.8 %) were recorded to be positive for *Mycobacterium bovis* by RD9 end-point PCR (Figure 1), all positive for *Mycobacterium bovis* based on the presence of RD1 and Ext-RD9 and on the absence of RD4, RD9 and RD12 markers, which represented 100 % concordance between methods. In this group, 50 % were found to be males and 50 % females. PPD was positive only in seven patients, with an average induration of 8 mm.

Figure 2. Posteroanterior chest X-rays of patients diagnosed with pulmonary tuberculosis by *Mycobacterium bovis*, where multiple lesions in cavens and alveolar occupation are observed.

By localization, 19 cases (39 %) were lymph node tuberculosis; 10 (21 %), pulmonary tuberculosis; nine (19 %), meningeal tuberculosis; seven (14 %), renal tuberculosis; two (4 %), peritoneal tuberculosis; and one (2 %), pleural tuberculosis. Average growth in the cultures was 31 days, and disease evolution was chronic, with an average of seven years.

Discussion

Mycobacterium bovis infection in domestic cattle persists in Mexico and represents a public health problem, unlike the countries of Western Europe, which have been declared free of bovine tuberculosis.⁹

In Mexico, the management of *Mycobacterium bovis* infection is regulated by Mexican Standard NOM-ZOO-031-1995. In Mexico, the magnitude of the problem of *Mycobacterium bovis* infection in the general population and in at-risk population is unknown; however, isolated investigations have indicated variations from 2 to 40 %.¹⁰ In Mexico and Latin America, as well as in other developing countries, the prevalence of *Mycobacterium bovis* disease is not documented or is underestimated.¹¹

Mycobacterium bovis infection has a broader spectrum of the disease, which causes for lung disease to progress to chronic stages, as we observed in our study: in the 10 patients with pulmonary tuberculosis, cavens, bronchiectasis and fibrothorax were identified, with a disease evolution of nine years (Figure 2).^{12,13}

Mycobacterium bovis tuberculosis occurs especially in a large number of domestic and wild mammals,

particularly in bovine livestock. According to location, cervical lymphadenopathy predominated in our series (39 %), which is consistent with other investigations reports. Evolution of the condition was four years, and in five patients, first-line treatment failed.^{13,14}

Spoligotype pattern comparisons have been carried out in Mexico, and they are consistent with those observed in cattle isolates; therefore, tuberculosis in humans caused by *Mycobacterium bovis* is derived from strains that circulate in bovine livestock.¹⁵ SB0120 is the most common spoligotype circulating on several continents, while SB0121 exists in Europe, Africa and America.¹⁶ The determination of *Mycobacterium bovis* most common spoligotype is the key to find the source of infection and control and prevent the disease.

The factors associated with higher prevalence of the disease are being an immigrant, Hispanic origin, female gender, use of glucocorticoids, low socioeconomic status and extrapulmonary presentations, which we corroborated in our study: mean age of the cases was 41 years, 45 % of the cases came from rural environments with low economic resources, eight cases had occupational exposure, 10 had HIV/AIDS with a CD4 count < 100 cells/mL.^{9,10} Unlike the results of other investigations, we did not record frequency differences with regard to patient gender.¹⁷

In emerging countries, 10 to 20 % of cases of tuberculosis are caused by *Mycobacterium bovis*; however, this percentage might be underestimated because most laboratories do not routinely perform mycobacterial cultures, and only few have systems to identify *Mycobacterium bovis*. There are few studies investigating genotypic diversity and drug resistance in *Mycobacterium bovis* from infections in animals or humans.¹⁸

In this study, we report the presence of *Mycobacterium bovis* in 10 % of the strains, which is a relevant result given the clinical repercussions it represents. In none of the 41 cases was *Mycobacterium bovis* infection suspected.¹¹

Current guidelines recommend longer treatment for tuberculosis caused by pyrazinamide-resistant organisms such as *Mycobacterium bovis*, in order to obtain a negative culture in sputum and a higher cure rate. In general, resistance to pyrazinamide has been reported in 38 % of cases in susceptible groups, with poor prognosis.¹⁹ In our series, 20 % of patients experienced relapse one year after treatment completion; three patients died four months after diagnosis.²⁰

In cultures identified as *Mycobacterium bovis*, a 206-bp amplification product was obtained in the RD9 PCR assay, which correlated in 100 % with the culture, biochemical tests and molecular tests results. Owing to its easiness, specificity and low cost, RD9 PCR is a tool of great utility for *Mycobacterium bovis* routine diagnosis or confirmation in tertiary care hospitals, unlike spoligotyping, which despite being regarded as an uncomplicated tool that generates reproducible results, is focused on epidemiological studies and large-scale sample processing, in addition to being a high-cost resource.⁸

Currently, it is essential to have new molecular biology methods in tertiary care reference hospitals that demonstrate their efficacy to identify different species of mycobacteria, such as multiplex PCR, which allows *Mycobacterium bovis* to be differentiated from *Mycobacterium tuberculosis* in sputum and extrapulmonary samples.^{20,21}

Molecular epidemiological studies are needed in medicine and in veterinary fields in order to define the importance of zoonotic tuberculosis in human tuberculosis, to know the route of transmission, the risk factors, the dominant spoligotypes among humans and animals and to understand the phylogeographic relationships of strains in Mexico.

Conclusion

Currently, *Mycobacterium bovis* is not thought of as the etiologic agent of pulmonary and extrapulmonary tuberculosis; however, it is important for rapid, sensitive and economic molecular biology diagnostic tests to be performed, in order to be able to start an adequate treatment. Collaboration between veterinary and public health services of the country is required in order to decrease the prevalence of this disease.

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