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# Identification of *Mycobacterium tuberculosis* complex based on amplification and sequencing of the *oxyR* pseudogene from stored Ziehl-Neelsen-stained sputum smears in Brazil

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**Abstract:** A cross-sectional analysis of stored Ziehl-Neelsen (ZN)-stained sputum smear slides (SSS) obtained from two public tuberculosis referral laboratories located in Juiz de Fora, Minas Gerais, was carried out to distinguish Mycobacterium *bovis* from other members of the *Mycobacterium tuberculosis* complex (MTC). A two-step approach was used to distinguish M. *bovis* from other members of MTC: (i) *oxyR* pseudogene amplification to detect MTC and, subsequently, (ii) allele-specific sequencing based on the polymorphism at position 285 of this gene. The *oxyR* pseudogene was successfully amplified in 100 of 177 (56.5%) SSS available from 99 individuals. No molecular profile of M. *bovis* was found. Multivariate analysis indicated that acid-fast bacilli (AFB) results and the source laboratory were associated ($p < 0.05$) with *oxyR* pseudogene amplification. SSS that were AFB++ SSS showed more *oxyR* pseudogene amplification than those with AFB0, possibly due to the amount of DNA. One of the two source laboratories presented a greater chance of *oxyR* pseudogene amplification, suggesting that differences in sputum conservation between laboratories could have influenced the preservation of DNA. This study provides evidence that stored ZN-SSS can be used for the molecular detection of MTC.
**Keywords:**. Tuberculosis; *M. bovis*; *M.* tuberculosis; *M. avium-intracellulare*; *oxyR* pseudogene; *pncA* gene; Zoonosis; Brazil

## Identificação do complexo *Mycobacterium tuberculosis* baseado na amplificação e sequenciamento do pseudogene *oxyR* de lâminas de baciloscopia coradas por Ziehl-Neelsen arquivadas no Brasil

**Resumo:** Uma análise transversal das lâminas de baciloscopia coradas por Ziehl-Neelsen (LB-ZN) arquivadas disponibilizadas foi realizada por dois laboratórios públicos de referência em tuberculose, localizados em Juiz de Fora, Minas Gerais, para distinguir *M. Bovis* de outros membros do complexo *Mycobacterium tuberculosis* (MTC). Uma abordagem de dois passos foi usada para distinguir *M. bovis* de outros membros do MTC: (i) amplificação do pseudogene *oxyR* para detectar MTC e, posteriormente, (ii) detecção do polimorfismo na posição 285 do pseudogene por sequenciamento. O pseudogene *oxyR* foi amplificado com sucesso em 100 de 177 (56,5%) LB-ZN disponíveis a partir de 99 indivíduos. Nenhum perfil molecular de *M. bovis* foi encontrado. A análise multivariada indicou que resultados de BAAR em cruzes e o laboratório fonte foram associados ($p < 0,05$) com a amplificação do pseudogene *oxyR*. Este estudo fornece provas de que armazenado LB-ZN podem ser utilizados para a detecção molecular de MTC.
**Palavras-chave**: Tuberculose*; M. bovis*, *M. tuberculosis*, *M. avium-intracellulare*; pseudogene *oxyR*; gene *pncA*; Zoonoses, Brasil.

## Introduction

Although most cases of human TB are caused by *Mycobacterium tuberculosis*, concerns over *M. bovis* have been expressed and are based on several observations. Firstly, occurrence of outbreaks of multidrug-resistant (MDR) *M. bovis* strains among hospitalized human immunodeficiency virus (HIV)-infected patients have been observed. This fact highlights the high spread risk of MDR *M. bovis*, especially in parts of Africa where *M. bovis* animal diseases and HIV human infection co-exist. Secondly, transmission from patients with pulmonary *M. bovis* disease to immune-competent contacts also appears to occur. Thirdly, the reemergence of human *M. bovis* carried by immigrants from regions where bovine tuberculosis is still prevalent have been documented in Europe and also on the United States-Mexico border. Finally, some 7,000 new cases of TB due to *M. bovis* may arise each year in Latin America (PAHO 1991).

Identification of mycobacteria by conventional biochemical identification methods is laborious and time-consuming and is not often performed by diagnostic laboratories. Therefore, other methods to differentiate *M. bovis* from other members of *M. tuberculosis* complex (MTC) that are faster and at least as equally or more sensitive than the classical methods are urgently needed. Such methods would aid in the identification and treatment of patients due to the intrinsic resistance of *M. bovis* to pyrazinamide (Konno et al. 1967) and for purposes of conducting epidemiological investigations.

Espinosa de los Monteros et al. (1998) described the use of an allele-specific polymerase chain reaction (PCR) method for detecting polymorphisms in *oxyR* to quickly and easily differentiate *M. bovis* from MTC. They found that the system based on *oxyR* could differentiate all of the *M. bovis* strains tested (including those isolated from goats).

Genetic analyses for the identification of mycobacteria species have been more common from isolates, but were rarely used in primary biological specimens or stored materials [sputum smear slides (SSS) or paraffin blocks]. The systems based on amplification and sequencing of the *oxyR* pseudogene from stored Ziehl-Neelsen (ZN)-stained SSS for identifying strains and distinguishing *M. bovis* from other members of the MTC have been implemented by research laboratories in Brazil. Public health programs and epidemiological studies may benefit from such methods.

This paper presents exploratory data describing the characterization of mycobacteria by molecular methods using DNA extraction, amplification and sequencing of the *oxyR* pseudogene using stored ZN-stained SSS obtained from patients with TB diagnosed at two public referral laboratories from Juiz de Fora, state of Minas Gerais (MG).

## Patients, Materials and Methods

Laboratory 1 is located in a TB regional hospital and performs SSS for hospitalized patients only, while laboratory 2 is a local referral centre and performs SSS in response to outpatient demands.

*Preparation and packing of SSS by laboratories* - The preparation of the SSS (smear preparation, fixation and staining of smears) by two involved laboratories was in accordance with the Tuberculosis Guidelines, Ministry of Health (2005). Both laboratories used blank glass slides for preparing the SSS. Following a microscopic exam, these slides were separated with tissue paper and stored at room temperature.

*DNA extraction* - The available SSS were sent to the Laboratory of Molecular Biology Applied to Mycobacteria at Oswaldo Cruz Foundation (Fiocruz, Rio de Janeiro, Brazil) for DNA extraction, amplification and sequencing of the *oxyR* pseudogene.

The *OxyR* pseudogene amplification was performed. A fragment of 150 bp was amplified. The bands generated were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide fluorescence.

*DNA sequencing of the oxyR locus* - We used sequencing of the *oxyR* locus to detect the polymorphism at position 285 of this gene.

Finally, the percentage of *oxyR* pseudogene amplification was estimated among the population, while factors associated with amplification were assessed using logistic regression models for both univariate and multivariate analysis. The difference in proportions was assessed using the Chi-square test and the magnitude of the associations was estimated by the odds ratio (OR) with 95% confidence interval (CI). The level of significance was 0.05.

## Results and Discussion

Descriptive characteristics of the population - Figure shows a flowchart conducting from the initial 177 specimens to the final selection of 93 slides from 93 valid source patients and the distribution of their frequencies of *oxyR* pseudogene amplification stratified by laboratories and positive/negative AFB smear microscopy results.

Among all of the 93 AFB results selected, 83 (89.3%) were positive and 10 (10.7%) were negative for AFB smear microscopy. The *oxyR* pseudogene was detected in 48 (57.8%) and six (60%) in each group, respectively. The frequency of *oxyR* pseudogene detection was higher among AFB-positive slides from laboratory 1 (84.6%) compared to laboratory 2 (34%). Among AFB0 (negative) slides, only the laboratory 1 had some amplification of the pseudogene *oxyR* (75%).

Finally, multivariate analysis indicated two variables that were statistically associated with presence of the *oxyR* amplification: AFB++ results by smear microscopy (OR = 8.06; 95% IC = 1.11-58.52) and SSS prepared in laboratory 1 (OR = 21.40; 95% IC = 5.95-76.90).

The independent association between AFB++ and the *oxyR* pseudogene amplification was evidenced in the multivariate analysis, a model that also took into account the laboratory that processed the SSS. Different amounts and varying preservation states of the DNA samples could more easily affect the amplification of single copy genes, such as *oxyR*, compared to multi copy genes.

In laboratory 1, the preparation of the SSS was done no later than 24 h after sputum collection. However, in laboratory 2, the sputum samples were sent from the primary health unit to a central unit where they were kept for a week before being forwarded to the laboratory where the SSS were prepared for microscopy. This excessive delay between sputum collection and smear preparation is of public health importance and quality assurance should be a concern in such settings.

## Conclusion

We have demonstrated the capability of amplifying and sequencing the *oxyR* pseudogene from stored SSS. However, we could not identify any molecular profile consistent with *M. bovis*.

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