Evaluation of PCR \textit{pncA}-restriction fragment length polymorphism and PCR amplification of genomic regions of difference for the identification of \textit{M. bovis} strains in lymph nodes cultures

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Abstract

Background: A rapid accurate identification of \textit{Mycobacterium bovis} is essential for surveillance purposes.

Objectives: A PCR \textit{pncA}-Restriction Fragment Length Polymorphism (RFLP) and a multiplex PCR based on the detection of 3 regions of difference (RD-PCR): RD9, RD4 and RD1 were evaluated for the identification of \textit{M. bovis} in lymph nodes cultures, in Tunisia, during 2013-2015.

Methods: Eighty-two \textit{M. tuberculosis} complex strains were identified using the biochemical tests, GenoType MTBC assay, PCR \textit{pncA}-RFLP and RD-PCR.

Results: The PCR \textit{pncA}-RFLP showed that 54 \textit{M. bovis} strains, identified by GenoType MTBC, had a mutation at position 169 of \textit{pncA} gene. Twenty-eight strains did not show any mutation at this position 27 \textit{M. tuberculosis} isolates and one \textit{M. caprae}. The PCR \textit{pncA}-RFLP had a sensitivity of 100.0\% (95\%CI: 93.3 -100.0) and a specificity of 100.0\% (95\%CI: 87.9-100.0) for identifying \textit{M. bovis}. The RD-PCR showed that all \textit{M. bovis} strains had the RD9 and RD4 deleted but presented RD1. RD-PCR also presented high sensitivity and specificity in detecting \textit{M. bovis} strains (100.0\%).

Conclusions: PCR \textit{pncA}-RFLP and RD-PCR represent very accurate and rapid tools to identify \textit{M. bovis}. They can be easily implemented in each laboratory due to their low cost and easy use.

Keywords: GenoType MTBC; lymph nodes; \textit{Mycobacterium bovis}; PCR \textit{pncA}-RFLP; RD-PCR.

DOI: https://dx.doi.org/10.4314/ abs.v21i3.4

Cite as: Bouzouita I, Draoui H, Mahdhi S, Essalah L, Sai- di I.S. Evaluation of PCR \textit{pncA}-restriction fragment length polymorphism and PCR amplification of genomic regions of difference for the identification of \textit{M. bovis} strains in lymph nodes cultures. Afri Health Sci. 2021;21(3): 985-989. https://dx.doi.org/10.4314/ abs.v21i3.4

Introduction

Zoonotic Tuberculosis (zTB) is caused principally by \textit{Mycobacterium bovis} and other species of \textit{Mycobacterium tuberculosis} complex (MTBC) e.g; \textit{M. caprae}, \textit{M. pinnipedii}, \textit{M. microti}, \textit{M. orygis}\textsuperscript{1,2,3,4,5}. The World Health Organization (WHO) estimates 147,000 new human cases in 2016 due to zTB with 12,500 deaths\textsuperscript{6}. In Tunisia, lymph node TB incidence was increased from 2.3 cases/100,000 inhabitants in 1993 to 18.0 cases/100,000 inhabitants in 2017 and \textit{M. bovis} could be responsible for 78.9\% of lymph node TB cases\textsuperscript{7}. \textit{M. bovis} is intrinsically resistant to pyrazinamide (PZA) due to the mutation C169G of \textit{pncA} gene (codon 57:H57D)\textsuperscript{8}.

Phenotypic and biochemical tests traditionally used to identify this species are time-consuming and inaccurate\textsuperscript{9}. The WHO recommended identifying this species to estimate the burden of zTB in each setting and prescribe an adequate treatment\textsuperscript{1}. Various methods have been developed for this purpose.

Sequencing based genotyping methods have been used as a reference standard to well differentiate between MTBC species. A set of molecular markers has been used for this aim, such as \textit{16S rRNA}, \textit{oxyR}, \textit{katG}, \textit{pncA}, \textit{gyrA}, \textit{gyrB} and \textit{hsp65}\textsuperscript{10-11}. However, sequencing-based genotyping methods are expensive and require specific equipment.

At the national reference laboratory (NRL) for mycobacteria in Tunisia, the line probe assay: Genotype
MTBC (Hain Lifescience, Germany) is used for molecular identification of *M. bovis* strains, whereas, this method is costly (34 $ for one test)

Herein, two cost-effective PCR approaches are evaluated: a PCR *pncA*-Restriction Fragment Length Polymorphism (RFLP) and a multiplex PCR based on the detection of three Region of Difference (RD9, RD4 and RD1) for the detection of *M. bovis* in lymph nodes cultures, in comparison with the line probe assay: GenoType MTBC assay.

**Materials and methods**

**Ethical approval**

This study is approved by the ethics committee of A. Mami pneumology hospital, Ariana, Tunisia.

**Clinical specimens, strains identification and phenotypic Drug susceptibility testing (DST)**

Two hundred sixty-four lymph nodes samples (n=264) were tested at the NRL for mycobacteria in Tunisia, during 2013- 2015. They were subjected to: acid-fast bacilli smear examination, a culture in liquid medium Mycobacteria Growth Indicator Tube 960 “MGIT960” (BD, USA), a culture in solid medium: Lowenstein Jensen “LJ”, and a molecular diagnosis by GeneXpert MTB/RIF (Cepheid, USA).

MTBC species identification was carried out by SD TB Ag MPT64 Rapid kit (Standard Diagnostics, South Korea), biochemical tests: niacin production, nitrate reductase activity, growth on thiophene-2-carboxylic acid hydrazide and the molecular assay GenoType MTBC.

To study the specificity of evaluated methods, different MTBC species selected from our strains bank: *M. caprae*, *M. bovis*, *M. bovis* BCG and *M. tuberculosis* H37Rv were included in addition to 7 species of non-tuberculous mycobacteria (NTM): *M. chelonae*, *M. abscessus*, *M. kansasii*, *M. intracellulare*, *M. fortuitum*, *M. marinum*, *M. peregrinum*. The NTM were identified by GenoType Mycobacterium CM/AS assay (Hain Lifescience, Germany).

**Data analysis**

Sensitivity, Specificity, Positive and Negative Predictive Values (PPV/NPV) were calculated using Open Epi version 3.01 with a confidence interval (CI) of 95%.

**Results**

During 2013-2015, lymphadenitis TB was confirmed in 164 cases (62.12%) by microscopy and /or culture and/or GeneXpert MTB/RIF. The culture was positive in 82 cases (50.0%). GenoType MTBC assay showed that TB lymphadenitis was due to *M. bovis* (n=54), *M. tuberculosis* (n=27) and *M. caprae* (n=1).

All *M. bovis* strains were resistant to PZA by MGIT960.

**Molecular identification by PCR pncA-RFLP**

PCR *pncA*-RFLP showed that 54 *M. bovis* strains presented 2 bands of 170 bp and 494 bp after digestion by BstEII (Figure 1). It showed that 28 strains presented 3 bands of 103bp, 170bp and 391bp (Figure 1). Twenty-seven were *M. tuberculosis* and one strain was *M. caprae*, according to GenoType MTBC.
PCR-RFLP had a sensitivity of 100.0% (95% CI: 93.3 -100.0), a specificity of 100.0% (95 CI: 87.9-100.0), a PPV of 100.0% (95% CI: 93.3 -100.0) and a NPV of 100.0% (95 CI: 87.9-100.0) for detecting *M. bovis*.

As regards the control strains: *M. bovis* and *M. bovis* BCG presented 2 bands after the digestion, whereas, *M. tuberculosis* H37Rv and *M. caprae* showed 3 bands. No amplification of *pncA* was detected for NTM species.

**Molecular identification by Regions of Difference**

All *M. bovis* strains (n=54) had RD9 and RD4 deleted. Our results showed that 27 strains presented the 3 RD targeted (RD9+/RD4+/RD1+) (Figure 2).

These strains belonged to (*M. tuberculosis*/ *M. canetti*) group. The biochemical tests and the GenoType MTBC identify these strains as *M. tuberculosis*. The *M. caprae* strain showed the absence of RD9 and the presence of RD4 and RD1 and was classified in (*M. caprae*/ *M. africanum*/ *M. pinnipedii* and *M. microti*) group.

The sensitivity of RD-PCR for identifying *M. bovis* was 100.0% (95% CI: 93.3 -100.0) with a specificity of 100.0% (95% CI: 87.9-100.0).

As concerns the control strains: *M. tuberculosis* H37Rv had the 3 RD studied, *M. caprae* presented only RD9, *M. bovis* had RD9 and RD4 deleted and *M. bovis* BCG had the 3 RD deleted. No amplification was found for the NTM species.

**Discussion**

*Mycobacterium bovis* is an important cause of TB in humans. Accurate, rapid identification of this species is required to allow appropriate treatment and set a strategy to monitor the cattle’s disease. For this purpose, two cost-effective PCR approaches were evaluated in comparison with the molecular assay: GenoType MTBC.

The molecular identification based on the polymorphism at position 169 of *pncA* presented very high
sensitivity and specificity in detecting \textit{M. bovis} strains (100.0\%). This method could also represent a rapid tool to detect the natural resistance to PZA. In fact, it is known that three MTBC species are intrinsically resistant to this drug: \textit{M. bovis}, \textit{M. bovis BCG}, due to the \textit{pncA} C169G substitution and \textit{M. canettii}\textsuperscript{14,15}.

The allelic variation at \textit{oxyR} position 285 has also been proposed to differentiate \textit{M. bovis} from \textit{M. tuberculosis} but did not distinguish between \textit{BCG} and non-\textit{BCG} \textit{M. bovis} strains\textsuperscript{11,16,17}.

In addition, a multiplex PCR was tested to detect the presence or absence of 3RD: RD9, RD4 and RD1\textsuperscript{12,13}. The RDs represent the loss of genetic materials in \textit{M. bovis BCG} compared to \textit{M. tuberculosis} H37Rv genome\textsuperscript{11}. All \textit{M. bovis} strains in this study (n=54) had RD9 and RD4 deleted but presented RD1. Consequently, the RD-PCR showed excellent sensitivity and specificity (100.0\%) for identifying \textit{M. bovis} isolates.

Compared with the conventional methods, \textit{pncA}-RFLP and RD-PCR represent accurate and fast tools (few hours versus many weeks for biochemical tests) to identify and differentiate \textit{M. bovis} from other MTBC members and NTM species. Furthermore, they have a low cost compared to GenoType MTBC (1.8\$ versus 34\$ for one test) and do not require expensive equipment and reagents as sequencing.

This study had some limitations: first, the two methods were tested using MTBC isolates and were not evaluated directly in lymph node samples. Second, mutation \textit{pncA} C169G was also found in \textit{M. bovis BCG} strains\textsuperscript{8,14}. In addition, some PZA resistant \textit{M. tuberculosis} isolates could display a mutation at this position. Consequently, these strains could be misidentified by \textit{pncA}-RFLP as \textit{M. bovis}.

However, \textit{M. bovis} BCG is rarely isolated from lymph node samples. In addition, a recent study in Tunisia has not reported any mutation at this position in PZA resistant \textit{M. tuberculosis} isolates\textsuperscript{18}.

Finally, it was shown that some \textit{M. caprae} strains and some \textit{M. tuberculosis} isolates belonging to lineage 3 displayed the RD4 deleted\textsuperscript{2,19,20}. Despite this finding, RD4 cannot be ruled out until further genomic deletion will be found to well distinguish between these species\textsuperscript{19}.

\textbf{Conclusions}

\textit{pncA}-RFLP and RD-PCR represent a rapid, accurate tools to detect \textit{M. bovis} in tuberculosis lymph nodes cultures compared with phenotypic and biochemical tests. They could be implemented easily in each laboratory owing to their easy use and low cost, in comparison with the DNA strip assay: GenoType MTBC and sequencing.

\textbf{Conflicts of interest}

None declared.

\textbf{Funding}

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

\textbf{Author contributions}

Imen Bouzouita, (Ph.D): conception of the work, doing experiments, interpretation of data, drafting the work, final approval and agreement.

Henda Draoui: conception of the work, doing experiments, critical revising of the manuscript, final approval and agreement.

Samia Mahdhi: conception of the work, doing experiments, critical revising of the manuscript, final approval and agreement.

Leila Essalah: conception of the work, doing experiments, critical revising of the manuscript, final approval and agreement.

Leila Slim Saidi (Professor): conception of the work, critical revising of the manuscript, final approval and agreement.

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