

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

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Abstract

Background: A rapid accurate identification of *Mycobacterium bovis* is essential for surveillance purposes.

Objectives: A PCR *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 *M. bovis* in lymph nodes cultures, in Tunisia, during 2013-2015.

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

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

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

Keywords: GenoType MTBC; lymph nodes; *Mycobacterium bovis*; PCR *pncA*-RFLP; RD-PCR.

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Introduction

Zoonotic Tuberculosis (zTB) is caused principally by *Mycobacterium bovis* and other species of *Mycobacterium tuberculosis* complex (MTBC) e.g; *M. caprae*, *M. pinnipedii*, *M. microti*, *M. orygis*^{1,2,3,4,5}.

The World Health Organization (WHO) estimates 147,000 new human cases in 2016 due to zTB with 12,500 deaths⁶. 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 *M. bovis* could be responsible for 78.9% of lymph node TB

cases⁷. *M. bovis* is intrinsically resistant to pyrazinamide (PZA) due to the mutation C169G of *pncA* gene (codon 57:H57D)⁸.

Phenotypic and biochemical tests traditionally used to identify this species are time-consuming and inaccurate⁹. The WHO recommended identifying this species to estimate the burden of zTB in each setting and prescribe an adequate treatment¹. 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 16S rRNA, *oxyR*, *katG*, *pncA*, *gyrA*, *gyrB* and *hsp65*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

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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). The phenotypic DST for first-line drugs was performed on MGIT 960 or LJ. For PZA, it was performed on MGIT 960 PZA kit (BD, USA).

PCR *pncA*-RFLP and RD-PCR

DNAs were extracted from MGIT 960 cultures. One ml of MGIT was centrifuged at 12.000 rpm for 10 min. The pellets were suspended in 200 µl of Tris EDTA Buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and heated at 95°C for 30 min. The suspensions were then cen-

trifuged at 13,000 rpm for 15 min and the supernatants were kept and frozen at -20°C.

The PCR mixture (25µl) for PCR *pncA*-RFLP method was prepared using 2.5 µl of buffer (10×), 0.1 µl of primers *pncA* F et R (25µM)¹¹, 2 µl of dNTP (10 mM), 2.5 µl of MgCl₂ (25 mM), 2.5 µl of DNA, 0.15 µl of Bioamtk Taq polymerase (500U) and water. The amplification was performed, according to Huard et al.¹¹. The PCR products (664bp) were digested by *BstEII* enzyme (New England, UK).

If 2 bands were obtained (170 bp and 494 bp): a mutation at position 169 of *pncA* is present.

If 3 bands were found (103 bp, 170 bp, and 391 bp): no mutation at position 169 of *pncA* gene.

For the RD-PCR method, the mix (25 µl) was composed of 2.5 µl of buffer (10×), 0.5 µl of primers F, R and int for each region RD1, RD4 and RD9 (25 µM)^{12,13}, 4 µl of dNTP (10 mM), 1 µl of MgCl₂ (50mM), 2.5 µl of DNA, 0.15 µl of Platinum Taq polymerase (Invitrogen, USA) and water.

The amplification was performed, according to Warren et al.¹³. The size of the bands, after electrophoresis, allows to deduce the absence (-) or presence (+) of the target regions: RD1+: 146bp; RD1-: 196bp; RD4+: 172bp; RD4-: 268bp; RD9+: 235bp; RD9-: 108bp¹³.

The species of MTBC, including *M. bovis*, were identified based on the presence or absence of these 3 RD.

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.

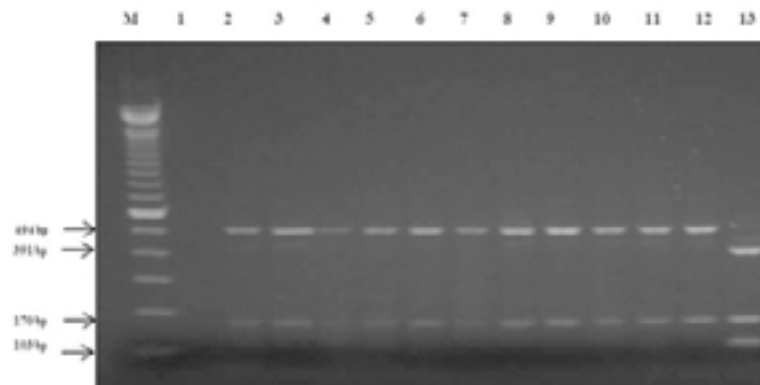


Figure 1. Results of PCR *pncA*-RFLP
M: 100bp DNA ladder, 1: *M. kansasii*(NTM: control strain), 2, 3, 4, 5, 6, 7, 8, 9, 10, 11: *M. bovis*, 12:*M. bovis* BCG (control strain), 13: *M. tuberculosis*

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).

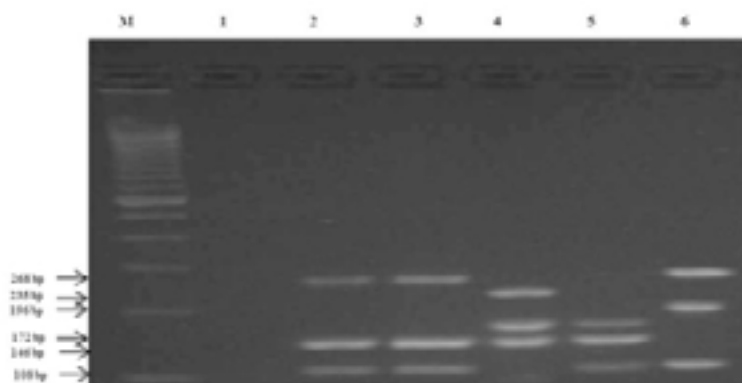


Figure 2. Results of the identification by RD
M: 100bp DNA ladder, 1*M. kansasii* (NTM: control strain), 2 and 3 *M. bovis*: 268bp (RD4-), 146bp (RD1+), 108bp (RD9-), 4 *M. tuberculosis*: 235bp (RD9+) 172bp, (RD4+), 146 bp (RD1+), 5*M. caprae*:172 bp (RD4+), 146bp (RD1+), 108bp (RD9-), 6 *M. bovis* BCG (control strain): 268bp (RD4 -), 196bp (RD1-), 108 bp (RD9-)

These strains belonged to (*M. tuberculosis/ M. canettii*) 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 *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: *M. bovis*, *M. bovis* BCG, due to the *pncA* C169G substitution and *M. canettii*^{14,15}.

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

In addition, a multiplex PCR was tested to detect the presence or absence of 3RD: RD9, RD4 and RD1^{12,13}. The RDs represent the loss of genetic materials in *M. bovis* BCG compared to *M. tuberculosis* H37Rv genome¹¹. All *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 *M. bovis* isolates.

Compared with the conventional methods, *pncA*-RFLP and RD-PCR represent accurate and fast tools (few hours versus many weeks for biochemical tests) to identify and differentiate *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 nodes samples. Second, mutation *pncA* C169G was also found in *M. bovis* BCG strains^{8,14}. In addition, some PZA resistant *M. tuberculosis* isolates could display a mutation at this position. Consequently, these strains could be misidentified by *pncA*-RFLP as *M. bovis*.

However, *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 *M. tuberculosis* isolates¹⁸.

Finally, it was shown that some *M. caprae* strains and some *M. tuberculosis* isolates belonging to lineage 3 displayed the RD4 deleted^{2,19,20}. Despite this finding, RD4 cannot be ruled out until further genomic deletion will be found to well distinguish between these species¹⁹.

Conclusions

pncA-RFLP and RD-PCR represent a rapid, accurate tools to detect *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.

Conflicts of interest

None declared.

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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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