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SHORT NOTE

Proposal of a new method for subtyping of *Mycobacterium kansasii* based upon PCR-restriction enzyme analysis of the *tuf* gene

**Running title:** New method for *Mycobacterium kansasii* subtyping

Zofia Bakula\(^1\), Magdalena Modrzejewska\(^1\), Aleksandra Safianowska\(^2\), Jakko van Ingen\(^3\), Małgorzata Proboszczy\(^2\), Jacek Bielecki\(^{1\#}\), Tomasz Jagielski\(^{1\#}\)

\(^1\)Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw, I. Miecznikowa 1, 02-096 Warsaw, Poland

\(^2\)Department of Internal Medicine, Pulmonology, and Allergology, Warsaw Medical University, Żwirki i Wigury 61, 02-091 Warsaw, Poland

\(^3\)Department of Medical Microbiology, Radboud University Medical Center, PO Box 9101, 6500HB Nijmegen, The Netherlands

**E-mail addresses:**

Zofia Bakula: zofiabakula@biol.uw.edu.pl
Magdalena Modrzejewska: magda.modrzejewska@wp.pl
Aleksandra Safianowska: aleksandra.safianowska@wum.edu.pl
Jakko van Ingen: vaningen.jakko@gmail.com
Małgorzata Proboszczy: m.proboszcz@wp.pl
Jacek Bielecki: jbielecki@biol.uw.edu.pl

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\(^\#\)**Corresponding author:** Tomasz Jagielski, PhD; Department of Applied Microbiology, Institute of Microbiology, Faculty of Biology, University of Warsaw; I. Miecznikowa 1; 02-096 Warsaw, Poland. Phone: +48 (0) 22 55 41 312; Fax: +48 (0) 22 55 41 402; E-mail: t.jagielski@biol.uw.edu.pl
Abstract

Within this study, a new, rapid method for subtyping of *Mycobacterium kansasii* was developed based on the sequence analysis of the *tuf* gene coding for the Tu (thermo-unstable) elongation factor (EF-Tu). The method involves PCR amplification of *ca.* 740-bp *tuf* gene fragment, followed by digestion with the MvaI restriction endonuclease.

**Keywords:** *Mycobacterium kansasii*; *tuf*; subtyping; PCR restriction-enzyme analysis

*Mycobacterium kansasii* is an opportunistic pathogen capable of causing severe lung disease, closely resembling pulmonary tuberculosis, as well as disseminated disease in the severely immunocompromised. It is among the most frequently isolated species of nontuberculous mycobacteria (NTM) (Hoefsloot et al., 2013; Braun et al., 2013). However, culturing *M. kansasii* from clinical samples of non-sterile sites need not necessarily represent true infection, but may result from occasional environmental contamination. Of the seven currently recognized *M. kansasii* subtypes (genotypes, I-VII) (Taillard et al., 2003) types I and II are most prevalent and have been associated with human disease, whereas the other five (III-VII) are predominantly of environmental origin or are believed to be environmental contaminants which sporadically cause disease (Bakula et al., 2013; Picardeau et al., 1997). Interestingly, *M. kansasii* type II has been found more likely to be recovered from patients with HIV-positive status, whereas type I has also been associated with pulmonary infections in HIV-negative patients with pre-existing pulmonary diseases (Tortoli et al., 1994).

Therefore, subtyping of *M. kansasii* isolates from human samples may serve as a proxy for clinical diagnosis.

Currently, there are two major methodologies used for the identification of *M. kansasii* subtypes I-VII. One is PCR-sequencing of either *hsp65* or *rpoB* genes, coding for the 65-kDa heat shock protein and the β-subunit of RNA polymerase, respectively or sequence analysis of
the 16S-23S rDNA internal transcribed spacer (ITS) (Iwamoto and Saito, 2005; Telenti et al., 1993; Kim et al., 2001). The other is PCR restriction-enzyme analysis (PCR-REA), which involves amplification of partial hsp65 or rpoB genes, followed by digestion of the amplicons with a combination of two or three restriction enzymes (BstEII and HaeIII for hsp65, and MvaI, AccII, HaeIII for rpoB) (Telenti et al., 1993; Kim et al., 2001).

Quite recently, the tuf gene, coding for the Tu (thermo-unstable) elongation factor (EF-Tu) has been exploited as a useful marker for the taxonomic classification of mycobacteria (Mignard and Flandrois, 2007). This study was undertaken to investigate whether the tuf gene can be applicable for subtyping of M. kansasii. For this purpose, the nucleotide sequences of the partial tuf gene from representatives of the M. kansasii subtypes I-VI were determined.

On the basis of the obtained data, a new PCR-REA assay was proposed, which allows for differentiation of M. kansasii subtypes with the use of only one restriction enzyme.

The following bacterial strains, representatives of six M. kansasii subtypes were used in the study: ATCC12478, ATCC25221, NLA001000927; NLA001000449; NLA00100521; 1010001495 (n=6, type I); B11063838, B11073207, NLA0010011128, 1010001469 (n=4, type II); 1010001468 (type III); 1010001458 (type IV); 1010001454, 1010001493 (type V); NLA001001166 (type VI). The strains were either purchased from the ATCC collection or donated by the Department of Medical Microbiology, Radboud University Medical Center, The Netherlands. The following bacterial strains, representatives of six M. kansasii subtypes, determined as such with a method by Telenti et al. (1993), were used in the study:

ATCC12478, ATCC25221, NLA001000927; NLA001000449; NLA00100521; 1010001495 (n=6, type I); B11063838, B11073207, NLA0010011128, 1010001469 (n=4, type II); 1010001468 (type III); 1010001458 (type IV); 1010001454, 1010001493 (type V); NLA001001166 (type VI). The strains were either purchased from the ATCC collection or
donated by the Department of Medical Microbiology, Radboud University Medical Center, The Netherlands.

The study also included 80 randomly selected *M. kansasii* clinical isolates, collected over a 14-year period (2000-2013) at the Department of Internal Medicine, Pulmonology, and Allergology, Warsaw Medical University, and representing 44% (80/182) of all *M. kansasii* strains collected during that time. They were identified as *M. kansasii* by using high pressure liquid chromatography (HPLC) methodology, in accordance with the Centers for Disease Control and Prevention (CDC) guidelines [Butler et al., 1996].

The isolates were recovered from pulmonary samples of 80 unrelated patients which accounted for 77% (80/104) of patients from whom *M. kansasii* strains were isolated between 2000 and 2013 (46 women, 34 men, aged between 21-92 years; median age, 64.3 years).

Of these patients, 27 (33.8%) had true pulmonary *M. kansasii* disease, based on the criteria published by the American Thoracic Society ATS) (Griffith et al., 2007), 17 (21.2%) had probable pulmonary *M. kansasii* disease, yet with not all ATS criteria fulfilled, and 36 (45%) patients were considered not to have *M. kansasii* disease. The final categorization of the cases was decided by a long-experienced pulmonologist, after consultation with the head of the mycobacteriology laboratory (Department of Internal Medicine, Pulmonology, and Allergology, Warsaw Medical University).

All strains were cultured on Löwenstein-Jensen (L-J) medium. Genomic DNA was extracted with the cetyl-trimethyl-ammonium bromide (CTAB) method, as described elsewhere (van Embden et al., 1993).

Subtyping of *M. kansasii* strains was originally performed upon ITS sequencing and PCR-REA analysis for *hsp65* and *rpoB*, as previously described (Iwamoto and Saito, 2005; Telenti et al., 1993; Kim et al., 2001).
For the amplification of the tuf gene fragment (ca. 740 bp) of all tested M. kansasii strains a PCR protocol designed by Mignard et al. with T1 and T2 primers was used (Mignard and Flandrois, 2007). The PCR reactions were performed with a TopTaq Master Mix kit (Qiagen, Hilden, Germany), as recommended by the manufacturer, in 50-µL reaction mixtures containing ca. 10 ng of bacterial DNA. Purified PCR amplicons (Clean-Up, A&A Biotechnology, Gdynia, Poland) obtained from 15 representatives of six M. kansasii subtypes (Fig. 1) were sequenced using the same primers as those used for the amplification. Sequence data were assembled and analyzed with ChromasPro (ver. 1.7.1, Technelysium, South Brisbane, Australia).

Searching for restriction sites was performed using insilico.ehu.es software (http://insilico.ehu.es/restriction/compare_seq/) (San Millan et al., 2013).

For all strains under the study, including 15 reference strains and 80 clinical isolates, the typing results obtained with PCR-REA of hsp65 and rpoB genes and sequencing of the ITS region were in 100% agreement. Of the 80 routine clinical isolates, all but one were categorized as subtype I. One strain was defined as M. kansasii subtype II. Computer-assisted analysis of the tuf gene revealed marked variations. The number of nucleotide differences between the tuf sequences ranged from 14 (subtype II versus IV) to 46 (subtype III versus VI), which translated into 93 to 98% sequence similarity, respectively. Noteworthy, all subtype I sequences (from 20 isolates in total) were identical. Likewise, all subtype II sequences (5 isolates) were identical. The similarity between two type V strains was 99% (3 nucleotide changes including one insertion). The tuf gene sequence from M. kansasii subtype VI shared the least similarity with those from other five subtypes (i.e. from 36 to 46 nucleotide changes or 93 to 94% sequence similarity, when compared to type IV and III, respectively) (Fig. 1).
FIG 1. A dendrogram (the Maximum Likelihood tree generated using the Tamura-3-parameter model in MEGA6 software) illustrating the genetic distances between different *M. kansasii* types (I-VI), based on the sequencing results of the partial *tuf* gene.

The obtained nucleotide sequences were searched for restriction sites which would yield the most discernible digestion fragments among the tested *M. kansasii* subtypes (I-VI). *In silico* simulations of restriction digestions were performed, and MvaI, the only one enzyme which produced distinct patterns for each *M. kansasii* subtype was selected for PCR-REA assay (Table 1).

### Table 1. Differentiation of *Mycobacterium kansasii* to subtype level with PCR-REA of the *tuf* gene using MvaI digestion enzyme.

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Fragments length (bp) designed in silico</th>
<th>Fragments length (bp) expected on agarose gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>321, 87, 84, 70, 69, 59, 51</td>
<td>320, 90, 70, 60, 50</td>
</tr>
<tr>
<td>II</td>
<td>321, 121, 87, 84, 69, 58</td>
<td>320, 120, 90, 70, 60</td>
</tr>
<tr>
<td>III</td>
<td>321, 171, 71, 69, 58, 51</td>
<td>320, 170, 70, 60, 50</td>
</tr>
<tr>
<td>IV</td>
<td>492, 72, 63, 58, 51, 6</td>
<td>490, 70, 60, 50</td>
</tr>
<tr>
<td>V</td>
<td>390, 171, 71, 57, 51</td>
<td>390, 170, 70, 60, 50</td>
</tr>
<tr>
<td>VI</td>
<td>408, 120, 84, 72, 59</td>
<td>410, 120, 80, 70, 60</td>
</tr>
</tbody>
</table>

To verify the results of *in silico* analysis and evaluate potential of designed method for laboratory use, the amplicons representing partial *tuf* gene sequence from all tested strains (15 subtypes representatives and 80 routine clinical isolates) were subjected to digestion with
MvaI restriction endonuclease. Digestion was done with the MvaI FastDigest® restriction enzyme, under conditions recommended by the manufacturer (Thermo Fisher Scientific, Waltham, USA), and the DNA fragments were electrophoresed on 4% agarose gels, and visualized by staining with ethidium bromide and UV fluorescence. The fragments matched exactly those expected (Table 1). As shown in Fig. 2, each subtype produced a characteristic restriction profile. The results of \textit{M. kansasii} subtyping with ITS sequencing and PCR-REA of \textit{hsp65}, \textit{rpoB}, and \textit{tuf} genes were fully concordant (Table 2).

**FIG 2.** Results of \textit{tuf} PCR-REA profiling for representatives of \textit{M. kansasii} subtypes: I (ATCC12478); II (B11073207); III (1010001468); IV (1010001458); V (1010001454); VI (NLA001001166); Molecular weight marker – GeneRuler LowRange DNA Ladder.
Table 2. Comparison of the results of four tested assays used for *M. kansasii* typing.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>No. of strains</th>
<th><em>M. kansasii</em> type established by</th>
<th>PCR-REA of sequencing of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>hsp65</td>
</tr>
<tr>
<td>ATCC12478</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC25221</td>
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<td></td>
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<tr>
<td>NLA001000927</td>
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<td></td>
<td></td>
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<tr>
<td>NLA001000449</td>
<td></td>
<td></td>
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<tr>
<td>NLA00100521</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1010001495</td>
<td>85</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>POL1-9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>POL11-80</td>
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<td></td>
<td></td>
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<tr>
<td>B1106338</td>
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<td>NLA001001128</td>
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</tr>
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<td>II</td>
<td>II</td>
</tr>
<tr>
<td>POL10</td>
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</tr>
<tr>
<td>1010001468</td>
<td>1</td>
<td>III</td>
<td>III</td>
</tr>
<tr>
<td>1010001458</td>
<td>1</td>
<td>IV</td>
<td>IV</td>
</tr>
<tr>
<td>1010001454</td>
<td>2</td>
<td>V</td>
<td>V</td>
</tr>
<tr>
<td>1010001493</td>
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<tr>
<td>NLA001001166</td>
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<td></td>
<td>1</td>
<td>VI</td>
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</table>

Among the modalities currently used for the identification of NTM species, including *M. kansasii*, several approaches have gained wide acceptance, including PCR-sequencing (Iwamoto and Saito, 2005; Duan et al., 2015), real-time PCR (Bainomugisa et al., 2015), and PCR-REA assays (Telenti et al., 1993; Kim et al., 2001). Also DNA hybridization technology and commercially available products based on this approach as the AccuProbe (Gen- Probe, San Diego, USA) (Richter et al., 1999), the INNO LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) (Padilla et al., 2004), the Speed-oligo® Mycobacteria (Vircell, Santa Fé Granada, Spain) (Ramis et al., 2105), MycoID Plus Kit (Bio Concept Corporation, Taichung, Taiwan) (Chien et al., 2015) and the GenoType Mycobacterium CM/AS (Hain Lifescience, Nehren, Germany) (Padilla et al., 2004) have been applied for the identification of *M. kansasii*. However, none of these tests can distinguish between *M. kansasii* subtypes. The exception is the INNO LiPA kit, which distinguishes only subtypes I, II, and subtypes III-V, as a group. Of the two methods capable of dividing *M. kansasii* into
distinct subtypes, PCR-REA offers clear advantages over PCR-sequencing in terms of technical feasibility (no need for specialized equipment) and cost-effectiveness, and thus is still often preferred in laboratory use and for diagnostic purposes.

The utility of PCR-REA based on a 652-bp fragment of the *tuf* gene selected by Mignard and Flandrois (Mignard and Flandrois, 2007) in the identification of NTM species was first evaluated by Shin et al. (Shin et al., 2009). A designed algorithm could differentiate 41 mycobacterial species, except for those within the *M. tuberculosis* complex, by using only two restriction enzymes (HaeIII and MboI). The present study proves that the same region can be employed as a marker for discriminating between *M. kansasii* subtypes.

Some limitations of this study should be noted. Firstly, *M. kansasii* subtype VII was not included in the strain sample tested. This subtype has so far been reported only in one study from Switzerland (it was identified in three out of 191 patients) (Taillard et al., 2003). Secondly, we were able to test only few isolates belonging to subtypes III-VI. This is because strains representing these types are extremely rare. Furthermore, one has to be aware that the genetic heterogeneity of *M. kansasii* species is not limited to only seven major *M. kansasii* subtypes described. Previously performed pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) analysis of *M. kansasii* subtypes I-V have demonstrated polymorphisms within those subtypes (Picardeu et al., 1997; Gaafar et al., 2009; Zhang et al., 2004). Moreover, strains of *M. kansasii* have been described of unknown *hsp65* PCR-REA patterns (Iwamoto and Saito, 2005; Kwenda et al., 2015) or with discrepant typing results by *hsp65* PCR-REA and ITS sequencing. Among the latter are type IIb strains with ITS sequences specific for subtype II, yet with unknown *hsp65* PCR-REA patterns or intermediate type I strains whose ITS sequences are specific for subtype II, whereas *hsp65* PCR-REA profiles are specific for subtype I (Iwamoto and Saito, 2005). All these strains,
however, have only anecdotally been reported, and their clinical relevance seems to be marginal (Gaafar et al., 2009; Zhang et al., 2004).

To conclude, the present study offers a new molecular tool for the discrimination of *M. kansasii* subtypes, which is clinically relevant. The PCR-REA proposed here produce identical results as with the former typing variants but has benefits over the latter, as it involves one digestion reaction with one restriction enzyme instead of multiple digestions with different enzymes.

**Nucleotide sequence accession numbers**

The nucleotide sequences of the partial *tuf* gene for representatives of the six (I-VI) *M. kansasii* subtypes were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) under the following accession numbers: KT284325-KT284331.

**Acknowledgments**

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**REFERENCES**


Highlights

- In the presented study, on the basis of sequence analysis of the \textit{tuf} gene, from representatives of the \textit{M. kansasii} subtypes I-VI, we designed a new molecular tool for the subtypes discrimination.

- The proposed PCR restriction-enzyme analysis assay (PCR-REA) should provide a clinically useful method of identifying \textit{M. kansasii} subtypes since its application is relatively fast, inexpensive and simple to use.

- The main advantage of the proposed method over previously designed PCR-REA assays is the use of only one digestion enzyme (instead of two or three, as in previously designed and currently used assays).