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

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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.

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) (Braun et al., 2013; Hoefsloot et al., 2013). However, culturing *M. kansasii* from clinical samples of nonsterile sites need not necessarily represent true infection but may result from occasional environmental contamination. Of the 7 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 5 (III–VII) are predominantly of environmental origin or are believed to be environmental contaminants which sporadically cause disease (Bakuła 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 preexisting 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 2 major methodologies used for the identification of *M. kansasii* subtypes I–VII. One is PCR sequencing of either *hsp65* or *rpoB* gene, 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; Kim et al., 2001; Telenti et al., 1993). The other is PCR restriction enzyme analysis (PCR-REA), which involves amplification of partial *hsp65* or *rpoB* gene, followed by digestion of the amplicons with a combination of 2 or 3 restriction enzymes (BstEII and HaeIII for *hsp65* and MvaI, AccII, and HaeIII for *rpoB*) (Kim et al., 2001; Telenti et al., 1993).

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 applicative 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 1 restriction enzyme.

The following bacterial strains, representatives of 6 *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

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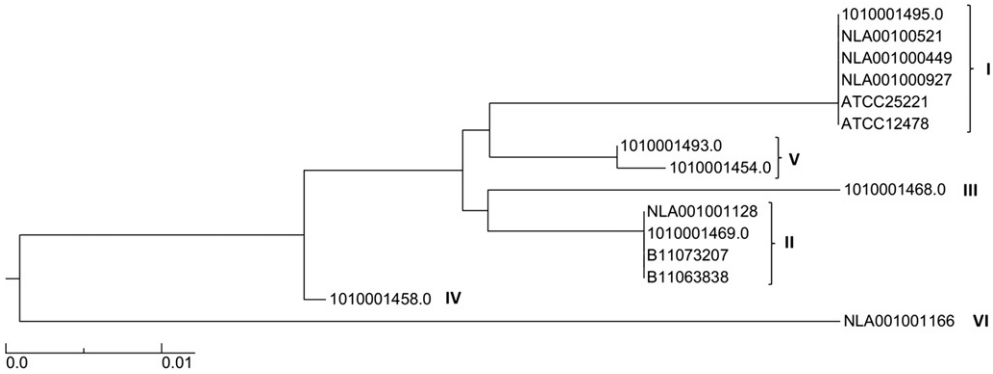

Fig. 1. A dendrogram (the maximum likelihood tree generated using the Tamura-3-parameter model in MEGA6 software) illustrating the genetic distances between different *M. kansasii* types (I–VI), based on the sequencing results of the partial *tuf* gene.

ATCC 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 and 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 medium. Genomic DNA was extracted with the cetyl-trimethyl-ammonium bromide method, as described elsewhere (van Embden et al., 1993).

Subtyping of *M. kansasii* strains was originally performed upon ITS sequencing and PCR-REA for *hsp65* and *rpoB*, as previously described (Iwamoto and Saito, 2005; Kim et al., 2001; Telenti et al., 1993).

For the amplification of the *tuf* gene fragment (*ca.* 740 bp) of all tested *M. kansasii* strains, a PCR protocol designed by Mignard and Flandrois (2007) with T1 and T2 primers was used. The PCRs 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 6 *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 Millán 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 1 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 93–98% sequence similarity, respectively. Noteworthy, all subtype I sequences (from 6 isolates in total) were identical. Likewise, all subtype II sequences (4 isolates) were identical. The similarity between 2 type V strains was 99% (3 nucleotide changes including 1 insertion). The *tuf* gene sequence from *M. kansasii* subtype VI shared the least similarity with those from other 5 subtypes (i.e., from 36 to 46 nucleotide changes or 93–94% sequence similarity, when compared to types IV and III, respectively) (Fig. 1).

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 1 enzyme which produced distinct patterns for each *M. kansasii* subtype, was selected for PCR-REA assay (Table 1).

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 subtype 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, MA, 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 *M. kansasii* subtyping with ITS sequencing and PCR-REA of *hsp65*, *rpoB*, and *tuf* genes were fully concordant (Table 2).

Among the modalities currently used for the identification of NTM species, including *M. kansasii*, several approaches have gained wide acceptance, including PCR sequencing (Duan et al., 2015; Iwamoto and Saito, 2005), real-time PCR (Bainomugisa et al., 2015), and PCR-REA assays (Kim et al., 2001; Telenti et al., 1993). Also, DNA hybridization technology and commercially available products based on this approach as the AccuProbe (Gen-Probe, San Diego, CA, USA) (Richter et al., 1999),

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

Subtype	Fragment length (bp)	
	Designed in silico	Expected on agarose gel
I	321, 87, 84, 70, 69, 59, 51	320, 90, 70, 60, 50
II	321, 121, 87, 84, 69, 58	320, 120, 90, 70, 60
III	321, 171, 71, 69, 58, 51	320, 170, 70, 60, 50
IV	492, 72, 63, 58, 51, 6	490, 70, 60, 50
V	390, 171, 71, 57, 51	390, 170, 70, 60, 50
VI	408, 120, 84, 72, 59	410, 120, 80, 70, 60