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ORIGINAL ARTICLE

Distribution of non-tuberculosis mycobacteria strains from suspected tuberculosis patients by heat shock protein 65 PCR–RFLP



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KEYWORDS

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Abstract The genus *Mycobacterium* contains more than 150 species. Non-tuberculosis mycobacteria (NTM) often cause extrapulmonary and pulmonary disease. Mycobacteria detection at species level is necessary and provides useful information on epidemiology and facilitates successful treatment of patients. This retrospective study aimed to determine the incidence of the NTM isolates and *Mycobacterium tuberculosis* (Mtb) in clinical specimens collected from Iranian patients during February 2011–December 2013, by PCR–restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene. We applied conventional biochemical test and *hsp65*–PRA identification assay to identify species of mycobacteria in specimens from patients suspected of having mycobacterial isolates. This method was a sensitive, specific and effective assay for detecting mycobacterial species and had a 100% sensitivity and specificity for Mtb and *Mycobacterium avium* complex (MAC) species. Using PRA for 380 mycobacterial selected isolates, including 317 Mtb, four *Mycobacterium bovis* and of the 59 clinical isolates, the most commonly identified organism was *Mycobacterium kansasii* (35.6%), followed by *Mycobacterium simiae* (16.9%), *Mycobacterium gordonae* (16.9%), *Mycobacterium fortuitum* (5.1%), *Mycobacterium intracellulare* (5.1%),

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Mycobacterium avium (5.1%), *Mycobacterium scrofulaceum* (3.4%), *Mycobacterium gastri* (3.4%), *Mycobacterium flavescens* (3.4%), *Mycobacterium chelonae* (3.4%) and *Mycobacterium nonchromogenicum* (1.7%). PRA method, in comparison with classical methods, is rapid, useful and sensitive for the phylogenetic analysis and species detection of mycobacterial strains. *Mycobacterium kansasii* is the most common cause of infection by NTM in patients with non-HIV and HIV which demonstrated a high outbreak and diversity of NTM strains in our laboratory.

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1. Introduction

The genus *Mycobacterium* contains more than 150 species (Tortoli, 2014). Non-tuberculosis mycobacteria (NTM), also using the name as environmental mycobacteria, atypical mycobacteria and mycobacteria other than tuberculosis (MOTT), are widely dispersed in the environment and can be found in soil, water, animals, unpasteurized milk and may cause disease in humans. Unlike *Mycobacterium tuberculosis* (Mtb), the majority of NTM infections come from the environment and is not spread from person to person. Exceptions include organisms in skin lesions, *Mycobacterium kansasii*, and maybe *Mycobacterium simiae* (Primm et al., 2004; Salvana et al., 2007).

The most common clinical presentation of NTM disease is pulmonary disease (90% of all NTM), but lymphatic, skin-soft tissue, and disseminated disease are also important. The incidence of chronic pulmonary disease, caused by NTM in human immunodeficiency virus (HIV)-negative patients, has also been increasing worldwide (Park and Kwon, 2014).

In HIV patients, there has been a dramatic increase in the number of diseases caused by the *Mycobacterium tuberculosis complex* (MtbC) and NTM in particular, especially members of the *Mycobacterium avium complex* (MAC) (Deepa et al., 2005).

At this moment, the Iranian reference laboratory has been reported (1980–1983) of which *Mycobacterium fortuitum* and *Mycobacterium kansasii* were recognized as the most dominant NTM in patients (Martin-Casabona et al., 2004). Up to now, several studies have attempted to detect the prevalence of NTM and its importance in Iran (Rahbar et al., 2010; Saifi et al., 2013; Shojaei et al., 2011).

The significant number of drug resistant patients such as multi-drug resistance (MDR), extensively drug resistant (XDR), and totally or extremely drug resistant TB (XXDR TB or TDR TB) tuberculosis may be non-tuberculosis mycobacteria (Shahraki et al., 2015).

Mycobacteria detection at species level is necessary and provides useful information on epidemiology and facilitates successful treatment of patients. Classical identification of mycobacteria based on cultural and biochemical tests is boring and needs more time. Several molecular assays such as PCR and other nucleic acid amplification methods are widely used to overcome these limitations. Among these various techniques, PCR–restriction fragment length polymorphism analysis (PCR–RFLP) is preferred because it offers a simple and inexpensive assay of identifying mycobacteria species in a single experiment. Moreover, the technique has been used to several genes, for example 16S ribosomal DNA (16SrRNA), heat-shock protein 65 (*hsp65* or *hsp60*), *dnaJ*, *groES*, 16S-23S

rRNA internal transcribed spacer (ITS) and DNA-directed RNA polymerase beta chain *rpoB* genes. The *hsp65* gene encoded a 65 kD heat-shock protein that is found in the cell wall of all species of *Mycobacterium* and include epitopes that are unique as well as common to different species of mycobacteria (Adékambi et al., 2003; Aravindhan et al., 2007; Deepa et al., 2005; Roth et al., 1998; Shinnick et al., 1988).

The aim of this study was to determine the incidence of the NTM and Mtb isolates by PCR–restriction fragment length polymorphism analysis (PRA) of the *hsp65* gene.

2. Materials and methods

2.1. Patients and mycobacterial strains

This retrospective study was conducted to investigate the clinical and microbiological features of infections caused by mycobacteria. All the 10,377 clinical samples including sputum, synovial fluid, urine, tissue biopsy and bronchial fluid, collected from Iranian patients suspected of having tuberculosis, referred to the Mycobacteriology Department of the Pasteur Institute of Iran during February 2011–December 2013, were examined. Demographic and clinical details of each patient were collected during sampling.

The current study was approved by Ethical Committee of Pasteur Institute of Iran. An informed consent was also directly obtained from each patient.

Specimens were processed by the standard N-acetyl-L-cysteine (NALC)–NaOH assay. Samples were processed using 2% NaOH/NALC method and concentrated at 3000 rpm for 20 min. The sediment was reconstituted to phosphate buffer (pH = 6.8), to make the inoculum for the fluorescent and Ziehl–Neelsen acid-fast microscopy, and cultures on Löwenstein-Jensen (L.J.) medium were incubated at 37 °C for at least 6 weeks (Kent et al., 1985).

2.2. Reference mycobacterial strains

The reference culture strains of Mtb (CRBIP7.11), *Mycobacterium bovis* (CRBIP7.121), *Mycobacterium scrofulaceum* (CRBIP7.163), *Mycobacterium avium* (CRBIP7.142), *Mycobacterium simiae* (CIP104531), *Mycobacterium chelonae* (CIP104535), *Mycobacterium kansasii* (CRBIP7.98), *Mycobacterium fortuitum* (CRBIP7.99), *Mycobacterium gastri* (CIP104530), *Mycobacterium flavescens* (CIP104533), *Mycobacterium xenopi* (CIP104035), *Mycobacterium goodii* (CIP104529), *Mycobacterium aurum* (CIP104482), *Mycobacterium intracellulare* (CRBIP7.168), *Mycobacterium nonchromogenicum* (CIP106811) and *Mycobacterium terrae* (CIP104321) were applied as comparison controls in order to

categorize the mycobacteria patterns in species identification grouping with the PRA method. Standard strains were provided from Pasteur Institute of France.

2.3. Biochemical tests

Detection of mycobacteria to species level was based on their growth rate, pigmentation and biochemical tests (macroscopic and microscopic morphological features, nitrate reduction test, niacin test, thiophene-2-carboxylic acid hydrazide test (TCH), semi-quantitative (sq) catalase and stability of catalase activity at 68 °C) according to the CDC guidelines (Kent et al., 1985).

2.4. DNA extraction

Mycobacterial DNA was extracted using a High Pure PCR Template Preparation kit (Roche, Germany). Purified DNA can be applied directly or stored at +2 to +8 °C for later analysis. DNA concentrations were detected by calculating the absorbance ratio OD₂₆₀ nm/280 nm using Thermo Scientific™ NanoDrop-2000 model (Thermo Scientific, Wilmington, DE).

2.5. Hsp65–PRA-based identification assay

An approximately 294-bp region of the *hsp65* gene was amplified by PCR using two specific primers HSP1 (5'-GCCAA GAAGACCGATGACGT-3') and HSP2 (5'-GGTGAT GACGCCCTCGTTGC-3') (Wong et al., 2001). The PCR was performed in a final volume of 25 µL, consisting of 5 µL of DNA, forwards and reverse primers (0.15 µM of each), different deoxynucleotides (0.2 mM of each), Ex Taq DNA Polymerase (1.5 U) (Takara Korea Biomedical Inc), and PCR buffer (10X) containing 1.5 mM MgCl₂.

First, the PCR was performed by denaturing the samples (for 7 min at 95 °C); then, 35 cycles including 95 °C for 45 s, 66 °C for 45 s, 72 °C for 45 s, and a final extension at 72 °C for 10 min were followed. Amplification of the 294-bp product of the *hsp65* gene was detected by 2% agarose gel electrophoresis.

2.6. Restriction fragment length polymorphism (RFLP)

Clinical and standard isolates were determined by using RFLP with the enzymes *Cfr13I* (*Sau96I*) and *BstHHI* (*CfoI*). PCR product (12.5 µL) was digested by 5 U of *Cfr13I* (Thermo Scientific™, USA) and *BstHHI* (Thermo Scientific™, USA) for 3 h at 37 °C. The digested products were visualized on 3% agarose gel electrophoresis at 100 V for 3 h. To interpret the PRA profiles generated by each species, a 50 bp ladder DNA size marker (Thermo Scientific™, USA) was applied. DNA fragments are visualized by DNA Safe Stainv2 (Sinaclon, Iran) and UV transilluminator.

3. Results

During the study period, a total of 10,377 clinical specimens were cultured for *mycobacterium*. Of these specimens (according to the biochemical tests), 317 samples with Mtb isolates (3.1%; 317/10,377) and 4 samples with *Mycobacterium bovis* isolates (0.04%; 4/10,377) were observed and 59 samples of NTM isolates were observed (0.56%; 59/10,377) (Fig. 1). The sources of the 59 NTM isolates were 56 respiratory specimens (sputum,

86.4% [51/59]; bronchial wash, 1.7% [1/59]; or gastric lavage, 6.8% [4/59]) and three nonrespiratory specimens (urine, 1.7% [1/59]; and skin lesion, 3.4% [2/59]).

In this study, according to patient's questionnaire, 317 Mtb isolated cases included 175 (55.2%) male and 142 (44.8%) female and four *Mycobacterium bovis* cases included 3 (75.0%) male and 1 (25.0%) female.

The average age of the Mtb isolated patients was 46.03 ± 10.9 years (range, 25–87 years) and *Mycobacterium bovis* patients was 48.5 ± 7.3 years (range, 41–57 years).

Signs and symptoms of Mtb, including coughing up sputum, weight loss, wheezing, fever, chest pain, hemoptysis and night sweats were observed in 52.2%, 42.5%, 36.7%, 36.5%, 25%, 17.5% and 14.2%, respectively.

Mtb isolates were non-pigmented, niacin positive, nitrate positive, resistant to TCH, stability of catalase activity at 68 °C and negative sq catalase and *Mycobacterium bovis* strains were negative for niacin accumulation and nitrate reduction and susceptible to TCH. Direct microscopic test among Mtb culture-positive samples was negative in 29.2%, whereas 70.8% were positive.

The average age of the patients (59 NTM isolated) was 50.9 ± 7.6 years (range, 25–61 years). Thirty-seven (62.7%) patients were male, and twenty-two (37.3%) were female. We had 11 (18.6%) HIV cases, in NTM patients, among our studied population. The most frequently isolated *mycobacterium* species were *Mycobacterium kansasii*, which was isolated in 5 (45.4%) out of 11 patients with NTM pulmonary disease, 4 (36.4%) patients had *Mycobacterium simiae* and 2 (18.2%) patients had *Mycobacterium avium*. Twenty-two (37.3%) out of 59 NTM isolates had negative results of direct microscopy smear.

A 294 bp PCR product among all 59 NTM and 321 MtbC isolates were amplified by the primers without any problems (Fig. 2).

All Mtb clinical isolates (317) and four *Mycobacterium bovis* strains were correctly identified by PRA and produced a similar pattern to *Mycobacterium bovis* (CRBIP7.121) and Mtb (CRBIP7.11) strains. Among complex isolates of Mtb, during the *Cfr13I* digestion experiment, two fragments of 219–240 and 75–54 bp were detected, while during *BstHHI* digestion, three different fragments, including 122, 83 and 72 bp, were observed (Fig. 2). Restriction fragments shorter than 50 bp were not analyzed, in order to increase the accuracy of strain detection.

The distribution of NTM species is summarized in Table 1. Of the 59 clinical isolates, the most commonly identified organism was *Mycobacterium Kansasii* (35.6%; 21/59), followed by *Mycobacterium simiae* (16.9%; 10/59), *Mycobacterium gordonae* (16.9%; 10/227), *Mycobacterium fortuitum* (5.1%; 3/59), *Mycobacterium intracellulare* (5.1%; 3/59), *Mycobacterium avium* (5.1%; 3/59), *Mycobacterium scrofulaceum* (3.4%; 2/59), *Mycobacterium gastri* (3.4%; 2/59), *Mycobacterium flavescens* (3.4%; 2/59), *Mycobacterium chelonae* (3.4%; 2/59) and *Mycobacterium nonchromogenicum* (1.7%; 1/59).

The PRA results were completely in agreement with those of the phenotypic biochemical test, except for one isolate, which was identified as *Mycobacterium chitae* using the PRA method but reported as *Mycobacterium flavescens* with biochemical tests (data not shown).

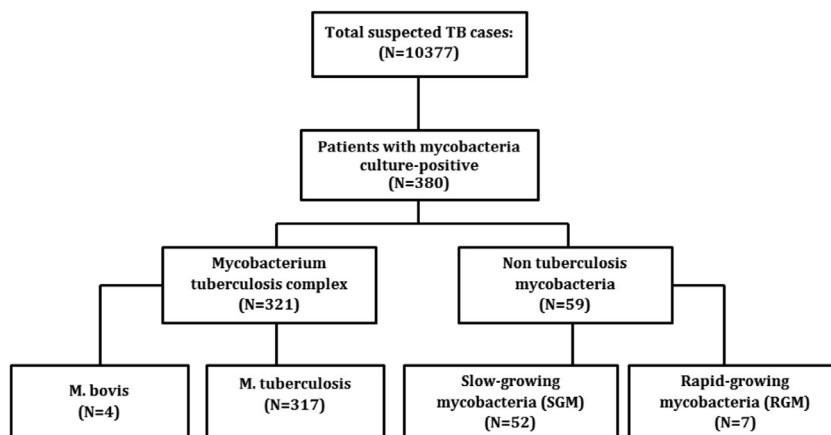


Figure 1 Flowchart of nontuberculous mycobacteria from culture-positive patients.

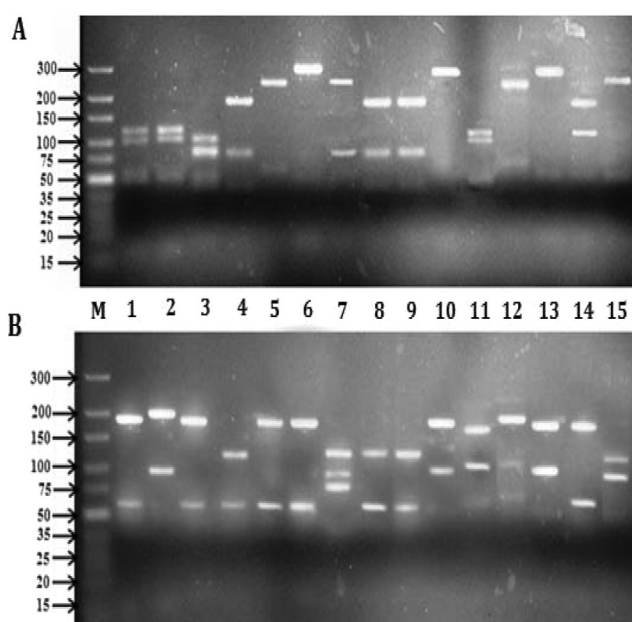


Figure 2 PRA patterns for different mycobacterial species. (A) *Cfr13I* digests; (B) *BstHHI* digests. M-DNA marker; 1-*Mycobacterium scrofulaceum*; 2-*Mycobacterium avium*; 3-*Mycobacterium simiae*; 4-*Mycobacterium gastri*; 5-*Mycobacterium chitae*; 6-*Mycobacterium xenopi*; 7-*Mycobacterium tuberculosis complex*; 8-*Mycobacterium kansasii*; 9-*Mycobacterium gordonae*; 10-*Mycobacterium aurum*; 11-*Mycobacterium intracellulare*; 12-*Mycobacterium nonchromogenicum*; 13-*Mycobacterium chelonae*; 14-*Mycobacterium terrae*; 15-*Mycobacterium fortuitum*.

4. Discussion

NTM are widely dispersed in the environment and can be found in soil, water, animals, and unpasteurized milk and may cause disease in humans (Salvana et al., 2007).

These bacteria are capable of causing severe disease in humans with a normal immune system and immunocompromised individuals but the majorities of NTM are less harmful and not virulent particularly (Velayati et al., 2015b).

However, in recent years, NTM are considered important opportunistic infections. In patients with NTM, clinical presentation is often difficult to differentiate from that of MTBC; hence, identification of NTM strains is very important, since the management and treatment of infected patients, as well as the epidemiological control assays implemented, must reflect the specific mycobacterial species encountered (Tortoli, 2014).

Phenotypic characterization including biochemical test properties and pigment production, growth rate, macroscopic and microscopic morphological features has been applied for the identification of *mycobacterium* species.

However, advantages of molecular techniques over conventional methods have been described in several studies to identify mycobacteria species (Chimara et al., 2008; Hashemi-Shahraki et al., 2013a; Kim et al., 2014; Kwon and Koh, 2014).

In this study, we used *hsp65* gene for the detection of mycobacteria species. The polymerase chain reaction and restriction enzyme analysis (PRA-*hsp65*) were carried out using the techniques described by Wong et al. (2001). In our study, this method was sensitive, specific, inexpensive and effective method for detection of mycobacterial species.

Wong and coworker have been reported that this method has the following properties: First, it has a 100% sensitivity and specificity for Mtb and MAC species. Second, except *Mycobacterium terrae* complex species, it gave favorably concordant results for other mycobacterial species. Third, “All mycobacterial isolates could readily be identified by eye, without computer assistance and it is highly suitable for large-scale use in a clinical laboratory” (Wong et al., 2001).

The most common genomic loci applied in molecular detection of NTM are the 16S-23S rRNA internal transcribed spacer (Hashemi-Shahraki et al., 2013a; Roth et al., 1998), *hsp65* (Jang et al., 2014; McNabb et al., 2006), 16S rRNA (Jing et al., 2014; Wu et al., 2014) and *rpoB* gene (Adékambi et al., 2003; Jang et al., 2014; Kim et al., 2014). To date, many studies have used multiple loci for the detection of NTM species, and *hsp65* is always included. McNabb et al. has shown a 99.1% concordance between biochemical methods or 16S rRNA gene sequencing compared to identification using *hsp65* gene sequencing, suggesting that *hsp65* gene (PRA method) is a sensitive, specific and effective assay for detecting mycobacterial species (McNabb et al., 2006).

Table 1 *Cfr13I* and *BstHHI* restriction patterns and results of mycobacterial strains identification by PRA assay.

Strain	<i>Cfr13I</i> (<i>Sau96I</i>)	<i>BstHHI</i> (<i>CfoI</i>)	Number (%)
<i>Mycobacterium tuberculosis</i> + <i>bovis</i>	219–240/54–75	122/83/72	317 + 4 (100)
<i>Mycobacterium kansasii</i>	183/75/36	122/57/50	21 (35.6)
<i>Mycobacterium gordonae</i>	183/75/36	172–180/53–63/39–50	10 (16.9)
<i>Mycobacterium simiae</i>	102/81/36–75	172–180/53–63/39–50	10 (16.9)
<i>Mycobacterium fortuitum</i>	219–240/54–75	122/83/50	3 (5.1)
<i>Mycobacterium intracellulare</i>	117/102/54–66	172/100	3 (5.1)
<i>Mycobacterium avium</i>	117/102/54–66	172–194/83	3 (5.1)
<i>Mycobacterium scrofulaceum</i>	117/102/54–66	172–180/53–63/39–50	2 (3.4)
<i>Mycobacterium flavescens</i>	219–240/54–75	172–180/53–63/39–50	2 (3.4)
<i>Mycobacterium gastri</i>	183/75/54	122/57/50	2 (3.4)
<i>Mycobacterium chelonae</i>	294	172/100	2 (3.4)
<i>Mycobacterium nonchromogenicum</i>	219–240/54–75	172–194/83	1 (1.7)
<i>Mycobacterium aurum</i>	294	172–194/83	0 (0)
<i>Mycobacterium terrae</i>	183/111	172–180/53–63/39–50	0 (0)
<i>Mycobacterium xenopi</i>	294	172–180/53–63/39–50	0 (0)

In our study, eleven ($n = 11$) different mycobacterial species other than Mtb were identified. According to the present study's results, NTM infections in our patients, including *Mycobacterium kansasii*, *Mycobacterium simiae*, *Mycobacterium gordonae*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium fortuitum*, *Mycobacterium scrofulaceum*, *Mycobacterium gastri*, *Mycobacterium flavescens* and *Mycobacterium nonchromogenicum*, using the two restriction enzymes, *Cfr13I* and *BstHHI* were determined. These results demonstrated a larger variation of NTM species in Iranian patients.

In this study, the most common type of NTM was *Mycobacterium kansasii* (35.6%). These results were consistent (Saifi et al., 2013) and inconsistent with a number of studies in Iran (Bahrmand et al., 1996; Tabarsi et al., 2015).

A relatively high incidence of *Mycobacterium kansasii* pneumonia has been reported in highly industrial areas with heavy air pollution (Arend et al., 2004). Of the 21 *Mycobacterium kansasii* isolates (all patients had pulmonary symptoms), 14 and 5 patients lived in Ahvaz and Tehran, respectively. According to the latest World Health Organization report, Ahvaz city has the world's worst air pollution (Masoudi et al., 2011).

In present study, *Mycobacterium simiae* (16.9%) was the second most prevalent NTM in clinical samples. The high frequency of *Mycobacterium simiae* isolate was reported from Iran. Many studies have been previously reported from Iran that the most frequently isolated NTM was *Mycobacterium simiae* (Baghaei et al., 2011; Hashemi-Shahraki et al., 2013b; Tabarsi et al., 2015).

In our study, *Mycobacterium gordonae* (likewise *Mycobacterium simiae*) was the second most frequently reported species.

Shojaei and coworkers have shown that the three most prevalent NTM isolates were *Mycobacterium fortuitum*, *Mycobacterium kansasii* and *Mycobacterium gordonae* (Shojaei et al., 2011).

Mycobacterium gordonae is widespread in the environment and considered a non-pathogenic isolate that it may cause contamination of clinical samples. *Mycobacterium gordonae* rarely causes true infection but the few cases of infection due to *Mycobacterium gordonae* were reported in immunosuppressed patients (Lessnau et al., 1993).

The different reports of NTM species in Iran might be related to different geographical conditions and studied populations. For example, *Mycobacterium gordonae* in Isfahan, *Mycobacterium simiae* in Tehran and *Mycobacterium intracellulare* in Khuzestan are commonly described (Velayati et al., 2015a).

In the current study, of the 59 NTM isolates, 11 patients had HIV infection. The most frequently isolated mycobacterium species was *Mycobacterium kansasii*, which was isolated in 5 (45.4%) of 11 patients with NTM pulmonary disease, 4 (36.4%) patients had *Mycobacterium simiae* and 2 (18.2%) patients had *Mycobacterium avium*.

Mycobacterium kansasii is the second-most-common NTM infection associated with HIV patients, outstripped only by MAC infection. The incidence of *Mycobacterium kansasii* infection increased with the expanding of the AIDS epidemic (Nguyen, 1997).

Four patients in the present study were HIV and *Mycobacterium simiae* positive. Three patients in our study were determined with Mtb and received anti-TB drugs for several years without showing any response to drugs. Thus, the rapid identification of NTM infection in HIV patients is important, particularly those caused by *Mycobacterium simiae*, and the significance of choosing the best treatment for non-responding TB patients should be taken into consideration.

Shojaei et al. has been reported that *Mycobacterium simiae*, are mainly problematic in an immunocompromised state for either HIV-positive or negative patients (Shojaei et al., 2011).

Two patients in the present study were HIV and *Mycobacterium avium* positive. *Mycobacterium avium* after Mtb is the most common mycobacterial cause of opportunistic in HIV patients, especially when CD4⁺ cell counts drop below 50 cell/ μ L (Corti and Palmero, 2008).

In endemic NTM regions such as Iran the PRA method has valuable potential because of its accurate clinical results that help in patient management and prevention plans. In developing countries such as Iran, where the prevalence of tuberculosis is high, because of the insufficiency of laboratory services, non-tuberculosis mycobacteria infections have developed (Saifi et al., 2013).

In Iran, the trends of isolation and detection of NTM have been enhanced in the last 20 years. This expanding trend is attributable to the implementation of increased molecular

methods that have enhanced the identification coupled with the enhanced consciousness of NTM in the clinical setting. Nevertheless, further research is required to address this important public health threat including enhancing the epidemiology of NTM throughout Iran, standardizing drug susceptibility testing and laboratory methods for identification, and improving clinician's knowledge on NTM diagnosis and treatment in Iran (Velayati et al., 2015a).

In conclusions, *Mycobacterium kansasii* is the most common cause of infection by NTM in patients with non-HIV and HIV which demonstrated a high outbreak and diversity of NTM strains in our laboratory. This method (PRA) was a sensitive, specific and effective assay for detecting mycobacterial species and had a 100% sensitivity and specificity for Mtb and MAC species.

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References

- Adékambi, T., Colson, P., Drancourt, M., 2003. RpoB-based identification of nonpigmented and late-pigmenting rapidly growing mycobacteria. *J. Clin. Microbiol.* 41, 5699–5708.
- Aravindhan, V., Sulochana, S., Narayanan, S., Paramasivan, C., Narayanan, P., 2007. Identification & differentiation of *Mycobacterium avium* & *M. intracellulare* by PCR–RFLP assay using the groES gene. *Indian J. Med. Res.* 126, 575–579.
- Arend, S., Cerda de Palou, E., De Haas, P., Janssen, R., Hoeve, M., Verhard, E., Ottenhoff, T., Van Soolingen, D., Van Dissel, J., 2004. Pneumonia caused by *Mycobacterium kansasii* in a series of patients without recognised immune defect. *Clin. Microbiol. Infect.* 10, 738–748.
- Baghaei, P., Tabarsi, P., Farnia, P., Marjani, M., Sheikholeslami, F. M., Chitsaz, M., Bayani, P.G., Shamaei, M., Mansouri, D., Masjedi, M.R., 2011. Pulmonary disease caused by *Mycobacterium simiae* in Iran's national referral center for tuberculosis. *J. Infect. Dev. Ctries.* 6, 23–28.
- Bahrmand, A.R., Samar, H.M.G., Khalilzadeh, L., Bakayev, V.V., Yaghlı, M., Babaei, M.H., 1996. Detection and identification of non-tuberculous mycobacterial infections in 6472 tuberculosis suspected patients. *Scand. J. Infect. Dis.* 28, 275–278.
- Chimara, E., Ferrazoli, L., Ueky, S.Y., Martins, M.C., Durham, A. M., Arbeit, R.D., Leão, S.C., 2008. Reliable identification of mycobacterial species by PCR-restriction enzyme analysis (PRA)–hsp65 in a reference laboratory and elaboration of a sequence-based extended algorithm of PRA–hsp65 patterns. *BMC Microbiol.* 8, 48.
- Corti, M., Palmero, D., 2008. *Mycobacterium avium* complex infection in HIV/AIDS patients.
- Deepa, P., Therese, K., Madhavan, H., 2005. Application of PCR-based restriction fragment length polymorphism (RFLP) for the identification of mycobacterial isolates. *Indian J. Med. Res.* 121, 694.
- Hashemi-Shahraki, A., Bostanabad, S.Z., Heidarieh, P., Titov, L.P., Khosravi, A.D., Sheikhi, N., Ghalami, M., Nojourni, S.A., 2013a. Species spectrum of nontuberculous mycobacteria isolated from suspected tuberculosis patients, identification by multi locus sequence analysis. *Infect. Genet. Evol.* 20, 312–324.
- Hashemi-Shahraki, A., Darban-Sarokhalil, D., Heidarieh, P., Feizabadi, M.M., Deshmir-Salameh, S., Khazae, S., Alavi, S.M., 2013b. *Mycobacterium simiae*: a possible emerging pathogen in Iran. *Jpn. J. Infect. Dis.* 66, 475–479.
- Jang, M.-A., Koh, W.-J., Huh, H.J., Kim, S.-Y., Jeon, K., Ki, C.-S., Lee, N.Y., 2014. Distribution of nontuberculous mycobacteria by multigene sequence-based typing and clinical significance of isolated strains. *J. Clin. Microbiol.* 52, 1207–1212.
- Jing, H., Tan, W., Deng, Y., Gao, D., Li, L., Lu, Z., Graviss, E.A., Ma, X., 2014. Diagnostic delay of pulmonary nontuberculous mycobacterial infection in China. *Multi. Respir. Med.* 9, 48.
- Kent, P.T., Kubica, G.P., Control, C.f.D., 1985. *Public health mycobacteriology: a guide for the level III laboratory*. US Department of Health and Human Services, Public Health Service, Centers for Disease Control.
- Kim, B.-R., Kim, J.M., Kim, B.-J., Jang, Y., Ryoo, S., Kook, Y.-H., Kim, B.-J., 2014. Identification of nontuberculous mycobacteria isolated from Hanwoo (*Bos taurus coreanae*) in South Korea by sequencing analysis targeting hsp65, rpoB and 16S rRNA genes. *Vet. Microbiol.* 173, 385–389.
- Kwon, Y.S., Koh, W.-J., 2014. Diagnosis of pulmonary tuberculosis and nontuberculous mycobacterial lung disease in Korea. *Tuberc. Respir. Dis. (Seoul)* 77, 1–5.
- Lessnau, K.-D., Milanese, S., Talavera, W., 1993. *Mycobacterium gordonae*: a treatable disease in HIV-positive patients. *Chest* 104, 1779–1785.
- Martin-Casabona, N., Bahrmand, A., Bennedsen, J., Østergaard Thomsen, V., Curcio, M., Fauville-Dufaux, M., Feldman, K., Havelkova, M., Katila, M., Köksalan, K., 2004. Non-tuberculous mycobacteria: patterns of isolation. A multi-country retrospective survey. *Int. J. Tuberc. Lung Dis.* 8, 1186–1193.
- Masoudi, M., Asadifard, E., Rastegar, M., 2011. Status and prediction of ozone as an air pollutant in Ahvaz City, Iran. *Caspian J. Environ. Sci.* 12, 215–224.
- McNabb, A., Adie, K., Rodrigues, M., Black, W.A., Isaac-Renton, J., 2006. Direct identification of mycobacteria in primary liquid detection media by partial sequencing of the 65-kilodalton heat shock protein gene. *J. Clin. Microbiol.* 44, 60–66.
- Nguyen, V., 1997. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. This official statement of the American thoracic society was approved by the board of directors, March 1997. Medical section of the American lung association. *Am. J. Respir. Crit. Care. Med.* 156, S1–25.
- Park, C.K., Kwon, Y.S., 2014. Respiratory review of 2014: tuberculosis and nontuberculous mycobacterial pulmonary disease. *Tuberc. Respir. Dis. (Seoul)* 77, 161–166.
- Primm, T.P., Lucero, C.A., Falkinham, J.O., 2004. Health impacts of environmental mycobacteria. *Clin. Microbiol. Rev.* 17, 98–106.
- Rahbar, M., Lamei, A., Babazadeh, H., Yavari, S.A., 2010. Isolation of rapid growing mycobacteria from soil and water in Iran. *Afr. J. Biotechnol.* 9, 3618–3621.
- Roth, A., Fischer, M., Hamid, M.E., Michalke, S., Ludwig, W., Mauch, H., 1998. Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *J. Clin. Microbiol.* 36, 139–147.
- Saifi, M., Jabbarzadeh, E., Bahrmand, A., Karimi, A., Pourazar, S., Fateh, A., Masoumi, M., Vahidi, E., 2013. HSP65–PRA identification of non-tuberculosis mycobacteria from 4892 samples suspicious for mycobacterial infections. *Clin. Microbiol. Infect.* 19, 723–728.
- Salvana, E.M.T., Cooper, G.S., Salata, R.A., 2007. *Mycobacterium* other than tuberculosis (MOTT) infection: an emerging disease in infliximab-treated patients. *J. Infect.* 55, 484–487.
- Shahraki, A.H., Heidarieh, P., Bostanabad, S.Z., Khosravi, A.D., Hashemzadeh, M., Khandan, S., Biranvand, M., Schraufnagel, D. E., Mirsaedi, M., 2015. “Multidrug-resistant tuberculosis” may be nontuberculous mycobacteria. *Eur. J. Intern. Med.* 26, 279–284.
- Shinnick, T.M., Vodkin, M., Williams, J., 1988. The *Mycobacterium tuberculosis* 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the *Escherichia coli* GroEL protein. *Infect. Immun.* 56, 446–451.

- Shojaei, H., Heidarieh, P., Hashemi, A., Feizabadi, M.M., Naser, A. D., 2011. Species identification of neglected nontuberculous mycobacteria in a developing country. *Jpn. J. Infect. Dis.* 64, 265–271.
- Tabarsi, P., Baghaei, P., Moniri, A., Marjani, M., Velayati, A.A., 2015. Nontuberculous mycobacterium in a tertiary tuberculosis center in Iran: dispensation and outcome of treatment. *Int. J. Mycobacteriol.* 4, 146.
- Tortoli, E., 2014. Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. *Clin. Microbiol. Rev.* 27, 727–752.
- Velayati, A.A., Farnia, P., Mozafari, M., Mirsaedi, M., 2015a. Nontuberculous mycobacteria isolation from clinical and environmental samples in Iran: twenty years of surveillance. *Biomed. Res. Int.* 2015, 10 254285.
- Velayati, A.A., Rahideh, S., Derakhshaninezhad, Z., Farnia, P., Mirsaedi, M., 2015b. Nontuberculous mycobacteria in Middle East: current situation and future challenges. *Int. J. Mycobacteriol.*
- Wong, D.A., Yip, P.C., Cheung, D.T., Kam, K.M., 2001. Simple and rational approach to the identification of *Mycobacterium tuberculosis*, *Mycobacterium avium* complex species, and other commonly isolated mycobacteria. *J. Clin. Microbiol.* 39, 3768–3771.
- Wu, J., Zhang, Y., Li, J., Lin, S., Wang, L., Jiang, Y., Pan, Q., Shen, X., 2014. Increase in nontuberculous mycobacteria isolated in Shanghai, China: results from a population-based study. *PLoS One* 9, e109736.