

Detection of Non-Tuberculosis Mycobacteria Infection due to *Mycobacterium leprae* and *Mycobacterium kansasii* in Patients Suspected of Tuberculosis in Isfahan, Iran

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ARTICLE INFO

Article history:

Received 10 January 2011

Accepted 7 February 2011

Available online 11 April 2011

Keywords:

Nontuberculosis mycobacteria,

rpoB gene,

Tuberculosis,

PCR-RFLP

ABSTRACT

Several reports have indicated that infection with Non-Tuberculosis Mycobacteria (NTM) is increasing worldwide. Therefore, monitoring species causing micobacterial infection in any region is of great importance. This study was going to detect, differentiate, and identify pathogenic *mycobacteria* in primary clinical samples. Eighty samples collected from tuberculosis suspected patients in Isfahan/Iran were included in this study. The clinical samples were processed for Acid Fast Bacilli (AFB), culture and PCR-PFLP procedures. A 342 bp fragment of *rpoB* gene was PCR amplified and the products were digested with *HindIII* restriction enzyme to discriminate between *tuberculosis* and *non-tuberculosis mycobacteria*. The PCR products were then digested with *HaeIII* restriction enzyme to identify the species. Of 80 studied samples, 8 showed AFB on microscopy, 9 were cultured positive for mycobacteria, and 32 (40%) were shown positive by PCR. Moreover, 2 specimens were infected with mycobacterium other than tuberculosis. Further digestion with the enzyme *HaeIII* showed that one of these samples was *Mycobacterium leprae* and the other one was *Mycobacterium kansasii*. The results obtained by this study show that similar to many other regions, nontuberculosis mycobacteria infection is increasing in the studied region, although its prevalence in Isfahan is yet lower than the southern parts of Iran.

1. Introduction

The genus *mycobacterium* includes 70 species, that causing serious human and animal disease and is considered as a major source of

morbidity and mortality worldwide (Kim et al., 1999; Haron et al., 2008).

The *mycobacterial* species that occur in humans and belong to the *M. tuberculosis* complex include *M. tuberculosis*, *M. bovis*,

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M. bovis BCG, and *M. africanum*. The term *nontuberculous mycobacteria* include all other *mycobacterial* species that do not belong to *M. tuberculosis complex*. Treatment is different for tuberculosis and NTM infections, and therefore, species identification is critical in patient management (Plikaytis et al., 1992).

Identification of a clinical isolate is primarily based on conventional methods such as culture characteristics, acid fast staining (Kinion, Ziehl, Nelsson) as well as biochemical tests (Betty et al., 2002). These methods take about 4 to 8 weeks to identify the species of *mycobacterium* and often are inconclusive (Inyaku et al., 1993; Deepa et al., 2005).

Various PCR-mediated methods have been developed for the rapid detection and differentiation of *mycobacterial* species. One of these methods is PCR amplification of a fragment of *rpoB* gene followed by restriction digest of the PCR products. This method proved to be useful not only for *mycobacterium* detection directly from *mycobacterial* DNA in clinical samples but also for its species identification (Kim et al., 1999; Lee et al., 2000; Kim et al., 2004; Gürtler et al., 2006; shin et al., 2008). It is a rapid, easy to perform and cost effective method that does not involve radioisotopes (Inyaku et al., 1993; Kim et al., 2004; Cheunoy et al., 2005; Varma-Basil et al., 2010).

In this study, we used *rpoB*-based PCR-RFLP method for identification and characterization of species responsible for *mycobacterial* infection. Here we report *mycobacterial* infection in 2 patients with *M. Lepra* and *M. Kasasii* NTM species. To the best of our knowledge it is the first report of human infection with these 2 species in the studied region.

2. Material and methods

2.1. Patients & clinical specimens

Eighty samples, prepared from those stored in the microbial collection laboratory of Shahid Sadoghi Hospital, Isfahan, Iran, were examined with three methods. The samples comprised 30 (37.5%) sputa, 18 (22.5%) urine, 14 (17.5%) pleural effusion, 2 (2.5%) neck, 4 (5%) aspirate cerebrospinal fluid (CSF), 2 (2.5%) prearticular

fluid, 2 (2.5%) acit fluid, 2 (2.5%) blood samples, and 6 (7.5%) tissue block.

2.2. Preprocessing of sputum and tissue block

Sputum samples were treated with 4% NaOH and 4% HCl, both for liquefaction and decontamination, followed by centrifugation at 12,000×g for 15 min to sediment bacterial cells. The tissue block samples were first deparaffinized by xylene treatment. The supernatant was removed and the pallet was re-suspended in 20µl proteinase K (10mg/ml) overnight at 37°C and then heated at 90 °C for 10 min. DNA of tissue samples were extracted using DNG Plus DNA extraction kits (Cinnagen; Tehran, Iran).

2.3. Staining and culture of samples

Smears were prepared from all samples and stained by Kinion, Ziehl, Nelsson method (carbon fuchsin for acid fast technique). The smears were then examined under oil immersion objective. The samples were then cultured in Lj. medium and the growth rate, pigment production by colonies, colony morphology, nitrate reduction and niacin were determined.

2.4. DNA extraction and amplification of the *rpoB* gene

For direct PCR, DNA was extracted from the samples. Clinical samples were incubated with 20 µl of 10 mg/ml proteinase K, overnight at 37 °C and then heated at 90 °C for 10 min. DNA of mycobacteria was extracted using DNG Plus DNA extraction kits (Cinnagen; Tehran, Iran). DNA concentration was determined by spectrophotometer. The supernatant were used as template for genus specific *rpoB* targeted PCR.

The following set of mycobacterium specific primers was used for PCR amplification: MF 5'-CGA CCA CTT CGG CAA CCG-3'; MR 5'-TCG ATC GGG CAC ATC CGG-3' (Boor et al., 1995; Kim et al., 1999; Kim et al., 2004). Each PCR reaction mixture contained 50 ng of template DNA, 20 pmol of each primer, 1 U of Taq DNA polymerase, 0.25mM of each dNTPs, and 1.5mM MgCl₂. The final volume adjusted to

25 µl with distilled water. The amplification protocol consisted of 35 cycles of denaturation at 95°C for 30 s, annealing at 58.5 °C for 30 s and extension at 72 °C for 45 s, followed by a final extension at 72 °C for 5 min, in an OmniGene thermocycler (Hybaid; Ashford, UK). Amplification of a 342 bp product of *rpoB* gene was evaluated by agarose gel electrophoresis (Electr-Y, Hybaid) and visualized with a UV transilluminator (Vilber Lourmat; Marne La Vallee, France).

2.5. Restriction analysis

In order to identify *M. tuberculosis* complex and NTM on basis of the RFLP pattern, positive product were digested for 2 h at 37 °C with *HindIII* restriction enzyme (Fig. 2). After the reaction, patterns were analyzed on a 6% polyacrylamid gel. Samples that were positive for NTM, were digested with *HaeIII* restriction enzyme, using the same condition as above.

3. Results

Based on *rpoB* gene region amplification, 32 (40%) clinical isolates were identified as

Mycobacterium tuberculosis (Fig. 1). The positive samples were from patients in age range between 1 to 80 years old, of which 23 (≈72%) and 9 (≈28%) samples were male and female, respectively. Using acid fast, bacteria (slender, curved rods and red to pink color) was seen only in 8 samples. Nine samples had Mycobacterial colony growth on Lj. media during 4 to 21 days with different morphology, pigment and other characters. All PCR positive samples produced an identical 342 bp band on agarose gel. Of the 32 positive clinical isolates, 30 proved to be *M. tuberculosis* complex and 2 were NTM, using *HindIII* restriction assay. One restriction site for this enzyme is present in the *rpoB* sequence of *M. tuberculosis* complex, producing 2 bands of 232 bp and 110 bp (Fig. 2). This restriction site, however, is not present in NTM strains.

In order to identify the species of these 2 NTM samples, the PCR products were restricted by *HaeIII* enzyme. By this method, one sample (Fig. 3, lane 8) produced three bands of 33 bp, 80 bp and 229 bp that are characteristic of *M. lepra*. The second sample (Fig. 3, lane 9) produced four bands of 29 bp, 34 bp, 50 bp and 229 bp, which is typical for *M. kansasii* (Kim et al., 2004).

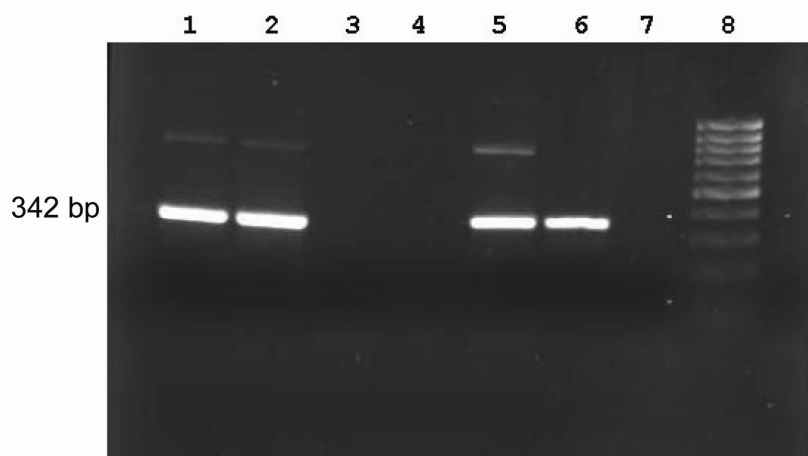


Fig. 1. Agarose gel (1.5%) analysis of a PCR diagnostic test for clinical sample. Lane1, 2, 5: positive clinical samples, Lane3, 4: negative clinical samples, Lane6: positive control, Lane7: negative control, Lane8: Molecular base pair standard (100-1000-bp ladder).

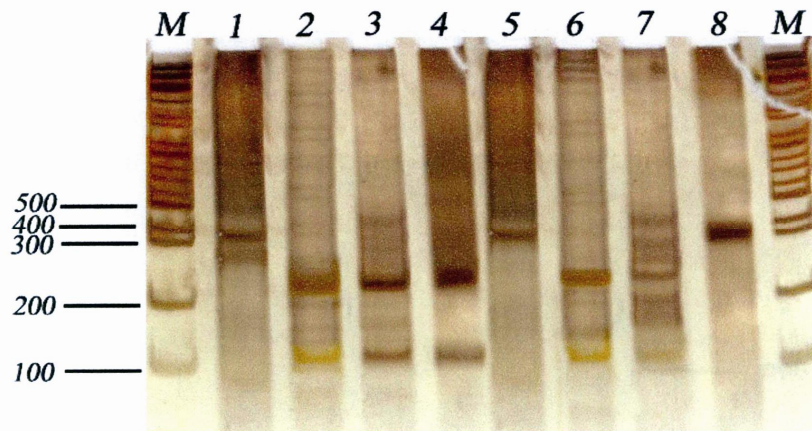


Fig. 2. Differentiation of *M. tuberculosis* complex from MOTT by the PRA of *rpoB* (*Hind*II). Amplified *rpoB* DNAs (342 bp) were digested with *Hind*II and electrophoresed on a 6% polyacrilamid gel. Only DNAs from the members of *M. tuberculosis* complex (lanes: 2, 3, 4, 6, 7) were digested (232 and 110 bp), while those of MOTT were not (lanes:1, 5, 8).

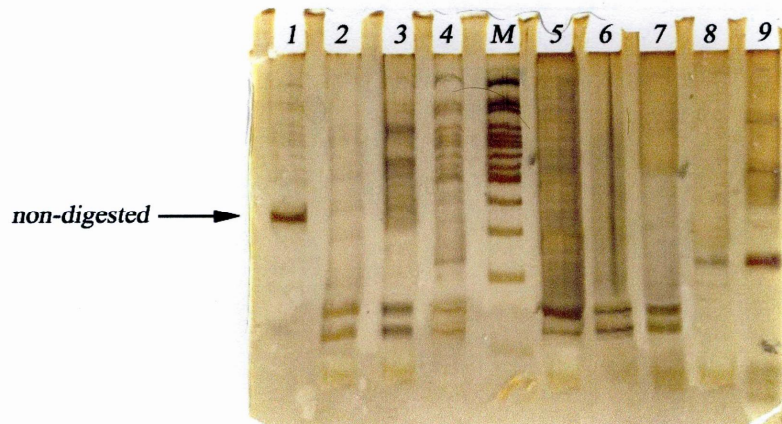


Fig. 3. Digestion of PCR products with *Hae*III. Lane 1, undigested sample; lanes 2 to 7, digested samples with 3 bands of 79 bp, 114 bp and 149 bp (*M. tuberculosis*); lane 8, digested sample with 3 bands of 33 bp, 80 bp, and 229 bp (*M. leprae* Thai 53 strain); lane 9, digested sample with 4 bands of 29 bp, 34 bp, 50 bp and 229 bp (*M. kansasii* subsp. III b).

4. Discussion

Micobacterial infection is considered as a threat to human health, worldwide. Identification and detection of mycobacterial species need rapid, reliable, specific and cost-effective techniques (Bannaliker and Verma, 2006; Khosravi et al., 2009). Conventional biochemical tests such as AFB staining and culture are not only time consuming but also unable to differentiate *M. tuberculosis* from NTMs in many cases (Bannaliker and Verma, 2006; Polanecky et al., 2006; Mokaddas and Ahmad, 2007). Our results as well as those

obtained by some other researchers show that PCR based methods could be more reliable in this regard. In a study by Banavaliker and colleagues, the sensitivity of PCR was reported to be nearly 100% in both smear and culture positive samples (Banavaliker et al., 1998).

In this study, the sensitivity of PCR was 100% in case of specimens determined positive by both smear and culture methods. Of 80 clinical specimens which included sputa, urine, pleural liquid, cerebrospinal fluid, prearticular fluid, neck sprite, acit fluid, blood, and tissue block, 17 specimens were positive for *M. tuberculosis* by culture and smear methods,

which *M. tuberculosis* DNA was detected in all of them by PCR. However, of 63 smear and culture negative samples, 15 were shown to be positive by PCR too.

Some researchers have recorded that differentiation between tuberculosis and NTM disease is not possible only by conventional AFB staining and conventional culture methods, and requires combining these methods with biochemical tests (Koh et al., 2005; Chimara et al., 2008). On the other hand, conventional methods are difficult and often fail to produce acceptable results. In a large study of 5000 clinical specimens, only 218 specimens were shown to be positive for *M. tuberculosis* by culture method, while *M. tuberculosis* DNA was detected in 85% of the specimens by PCR (Banavaliker et al., 1998). In another study, out of 313 clinical samples 95% of the smear positive and 57% of the smear and culture negative specimens were detected positive by PCR (Boor et al., 1995; Banavaliker et al., 1998). Pao et al. also detected *M. tuberculosis* DNA in 41.9% of specimens by PCR, while only 16% were found to be positive by culture method (Pao et al., 1998).

PCR-restriction enzyme analysis is a rapid and reliable method for differentiation between MT and NTM. In current study, the prevalence of NTM in samples with positive PCR was 6.25%. This result is similar to that reported by Khosravi et al. (2009). In a study done by Bannaliker and Verma (2006) 3 (10%) out of 30 samples were shown to be *M. avium*, while others were *M. tuberculosis* (Bannaliker and Verma, 2006). Another study carried out by Korean researchers from year 2004 until 2005, demonstrated that the prevalence of NTM was 24.4% in Korea (Park et al., 2006). In study done by Elazig Province, 51 Mycobacteria were isolated, of which 4 (7.8%), 2 (3.9%), and 1 (1.9%) were identified as *M. scrofulaceum*, *M. avium*, and *M. intracellulare*, respectively (Agacayak et al., 2007). The prevalence of NTM in Southeast of Iran has been reported as 33.3% (Naderi et al., 2006), while in Khuzestan, in Southwest of Iran, it is estimated to be 9.1% (Khosravi et al., 2009). The comparison between our results and those of the studies quoted above reveals that the prevalence of NTM in Isfahan,

located in center of Iran, is relatively lower than those in southern parts of the country.

In conclusion, findings of this study suggest that PCR-RFLP is a useful method for rapid detection of Mycobacteria directly from clinical specimens, and differentiation of *Mycobacterium tuberculosis* from NTM. Application of this method will be helpful for early diagnosis and treatment of Mycobacteria infections. Our results also are alarming for the local health system of the sampling area to pay more attention to the new species of Mycobacteria as a potential source of human infection.

References

- Agacayak, A., Bulut, Y., Seyrek, A., 2007. Detection of Mycobacterium species distribution in the sputum samples of tuberculosis patients by PCR-RFLP method in Elazig province. *Mikrobiyol Bul.* 41, 203-9.
- Banavaliker, J.N., Bobby, D.C., Manoj, K., Goel, P.S., Khandekar, P., Bose, M., 1998. Identification of Mycobacterium Tuberculosis by polymerase chain reaction in clinical Bannaliker, A.S., Verma, R., 2006. Detection of Mycobacterium avium & M. tuberculosis from human sputum cultures by PCR-RFLP analysis of hsp65 gene & pncA PCR. *Indian J Med Res.* 123, 165-172.
- Betty, A., Forbes Daniel, F., Sahn Alice, S., 2002. Mycobacteria and other Bacteria unusual growth requirements in: Nontuberculous Mycobacteria, *Diagnostic Microbiology*. Patricia Tannian. Andrew Allen., pp:554, ISBN-323-01678-2.
- Boor, K.J., Duncan, M., Price, C.W., 1995. Genetic and transcriptional organization of the region encoding the beta subunit of Bacillus subtilis RNA polymerase. *J Biol Chem.* 270, 20329-36.
- Cheunoy, W., Prammananan, T., Chaiprasert, A., Foongladda, D.S., 2005. Comparative evaluation of polymerase chain reaction and restriction enzyme analysis: two amplified targets, hsp65 and rpoB, for identification of cultured mycobacteria. *Diagn Microbiol Infect Dis.* 51, 165-71.
- Chimara, E., Ferrazoli, L., Ueky, S., Martins, M., Durham, A.M., Arbeit, R., 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.* 20 (8), 48.
- Deepa, P., Therese, K., Madhavan, H., 2005. Application of PCR-based restriction fragment length polymorphism for the identification of mycobacterial isolates. *Indian J Med Res.* 121, 694-700.
- Gürtler, V., Harford, C., Bywater, J., Mayall, B., 2006. Direct identification of slowly growing Mycobacterium

- species by analysis of the intergenic 16S-23S rDNA spacer region (ISR) using a GelCompar II database containing sequence based optimization for restriction fragment site polymorphisms (RFLPs) for 12 enzymes. *J Microbiol Methods*. 64, 185-99.
- Haron, S., Rahizan, I., Nik Marzuki, S., Noraziah, M., 2008. The Usefulness of PCR Amplification for Direct Detection of Mycobacterium tuberculosis DNA from Clinical Samples. *Biotechnology*. 7, 100-105.
- Inyaku, K., Hiyama, K., Ishioka, S., Inamizu, T., Yamakido, M., 1993. Rapid detection and identification of mycobacteria in sputum samples by nested polymerase chain reaction and restriction fragment length polymorphisms of dnaJ heat shock protein gene. *Hiroshima J Med Sci*. 42, 21-31.
- Khosravi, A.D., Seghatoleslami, S., Hashemzadeh, M., 2009. Application of PCR-Based Fingerprinting for Detection of Nontuberculous Mycobacteria among Patients Referred to Tuberculosis Reference Center of Khuzestan Province, Iran. *Res. J. Microbiol*. 4, 143-149.
- Kim, B.J., Lee, S., Lyu, M., Kim, S., Bai, G., Chae, G., Kim, E., Cha, C., Kook, Y., 1999. Identification of mycobacterial species by comparative sequence analysis of the RNA polymerase gene (rpoB). *J Clin Microbiol*. 37, 1714-20.
- Kim, B.J., Hong, S.K., Lee, K., Yun, Y.J., Kim, E.C., Park, Y.G., Bai, G.H., Kook, Y.H., 2004. Differential identification of Mycobacterium tuberculosis complex and nontuberculous mycobacteria by duplex PCR assay using the RNA polymerase gene (rpoB). *J Clin Microbiol*. 42, 1308-12.
- Koh, W.J., Kwon, O.J., Lee, K.S., 2005. Diagnosis and treatment of nontuberculous mycobacterial pulmonary diseases: a Korean perspective. *J Korean Med Sci*. 20, 913-25.
- Lee, H., Park, H.J., Cho, S.N., Bai, G.H., Kim, S., 2000. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the rpoB gene. *J Clin Microbiol*. 38, 2966-2971.
- Mokaddas, E., Ahmad, S., 2007. Development and evaluation of a multiplex PCR for rapid detection and differentiation of Mycobacterium tuberculosis complex members from non-tuberculous mycobacteria. *Jpn J Infect Dis*. 60, 140-4.
- Naderi, M., Alavi-Naini, R., Sharifi-Mood, B., Naserfar, M., 2006. Prevalence of Tuberculosis and Non Tuberculosis Mycobacterium in Zahedan, Southeast of Iran. *Res. J. Microbiol*. 1, 375-377.
- Park, C.M., Heo, S., Park, K.U., Song, J., Lee, J.H., Lee, C.T., Kim, E.C., 2006. Isolation of nontuberculous mycobacteria using polymerase chain reaction-restriction fragment length polymorphism. *Korean J Lab Med*. 26, 161-7.
- Plikaytis, B.B., Plikaytis, B.D., Yakrus, W.R., Butler, C.L., Woodley, V.A., Silcox, V., Shinnick, T.M., 1992. Differentiation of slowly growing Mycobacterium species, including Mycobacterium tuberculosis, by gene amplification and restriction fragment length polymorphism analysis. *J Clin Microbiol*. 3, 1815-22.
- Polanecky, V., Kalina, P., Kubin, M., Kozakova, B., Mullerova, M., 2006. Nontuberculous mycobacteria and incidence of mycobacterioses in Prague in 1999-2004. *Epidemiol. Mikrobiol. Imunol*. 55, 151-157.
- Shin, J.H., Lee, H., Cho, J., Yu, J.Y., Kang, Y.H., 2008. Targeting the rpoB gene using nested PCR-restriction fragment length polymorphism for identification of nontuberculous mycobacteria in hospital tap water. *J Microbiol*. 46, 608-14.
- Valente, C., Demas, S., Scarso, S., Cuteri, C., Job, S., Marangon, L., Cancellotti, F.M., 1997. The polymerase chain reaction for the identification of different species of Mycobacterium. *Comp Immunol Microbiol Infect Dis*. 20, 233-9.
- Varma-Basil, M., Pathak, R., Singh, K., Kumar Dhar, S., Dwivedi, K., Garima, S., Kumar, D., Sharma, B., Dhiman, M., 2010. Direct Early Identification of *Mycobacterium tuberculosis* by PCR-Restriction Fragment Length Polymorphism Analysis from Clinical Samples. *Jpn. J. Infect. Dis*. 63, 55-57.