



Research Journal of **Microbiology**

ISSN 1816-4935



Academic
Journals Inc.

www.academicjournals.com

**Application of PCR-Based Fingerprinting for Detection of Nontuberculous
Mycobacteria among Patients Referred to Tuberculosis
Reference Center of Khuzestan Province, Iran**

^{1,2}A.D. Khosravi, ¹S. Seghatoleslami and ¹M. Hashemzadeh

¹Department of Microbiology, School of Medicine,

²Infectious and Tropical Diseases Research Center,

Ahwaz Jondishapour University of Medical Sciences, Ahwaz, Iran

Abstract: The present study was conducted to determine the frequency of NTM by application of PCR-based Restriction Fragment Length Polymorphism (PCR-RFLP) among suspected tuberculosis patients. In total 150 clinical isolates from patients referred to TB reference laboratory were screened. Culture and biochemical tests were performed. The PCR-RFLP method based on amplification of a 439 bp fragment of *hsp* gene involving genus specific primers was performed and the PCR products were digested with *Hae* S and *Bst* EA restriction enzymes. Of total isolates tested, 100 isolates were culture positive (66.6%). Eighty out of 88 isolates that were subjected to RFLP, showed the identical restriction patterns similar to *Mycobacterium tuberculosis* (90.9%). Eight clinical isolates (9.1%) showed different restriction patterns, six isolates identified as *Mycobacterium intracellulare* and two isolates were *Mycobacterium gordonae* I. In conclusion, RFLP as a fast, cheap and accurate technique is a valid alternative for phenotypic identification of pathogenic and potentially pathogenic mycobacteria in the routine laboratory.

Key words: Fingerprinting, nontuberculous mycobacteria, PCR, RFLP, tuberculosis

INTRODUCTION

The genus *Mycobacterium* comprises more than 90 species that include pathogenic or potentially pathogenic species both for humans and animals. Among them the most important pathogens are the member of *Mycobacterium tuberculosis* complex and *Mycobacterium leprae* (Frieden *et al.*, 2003). Other members of the genus, however are usually saprophytes but can be opportunistic and are known as atypical mycobacteria or Non-Tuberculous Mycobacteria (NTM) (Ferdinand *et al.*, 2004). While *M. tuberculosis* the causative agent of tuberculosis in man, is transmitted through respiratory droplet inhalation, the NTM are thought to be acquired from the environmental sources such as soil or water and are often referred to as environmental mycobacteria (Jarzembowski and Young, 2008). NTM have been recognized since late in the 19th century but only since the 1950s have they been well recognized as a cause of human disease (Ferdinand *et al.*, 2004).

Before the AIDS epidemics, disease caused by NTM was quite rare and was restricted to pulmonary, cervical lymph nodes, skin and rarely was disseminated (Ferdinand *et al.*, 2004). By emergence of AIDS epidemic, the picture of NTM disease was changed. Although the most predominant *Mycobacterium* isolated from AIDS patients is *M. tuberculosis*, however, NTM species such as *M. avium* complex (MAC) are frequently cause disease in AIDS patients (Hernandez *et al.*, 1999). MAC infection in such cases could possibly occurs via the lungs as respiratory pathogen and

Corresponding Author: Azar D. Khosravi, Department of Microbiology, School of Medicine,
Ahwaz Jondishapour University of Medical Sciences, Ahwaz, Iran
Tel: +98 611 3330074 Fax: +98 611 3332036

may cause pulmonary fibrosis or cavity lung disease (Falkinham III, 1996). The infection could affect, gastrointestinal tract as well (Frieden *et al.*, 2003). Therefore due to increased morbidity and mortality which is associated with NTM infections and since there are a few clinical and radiological differential findings between NTM infections and tuberculosis, the precise and rapid diagnostic tests are needed for differentiation of NTM from *M. tuberculosis* and Identify NTM members to species level (Johansen, 2006). The distinction between species is important in view of epidemiological implications and patient's treatment management.

The traditional diagnosis of mycobacteria based on culture and biochemical identification tests, despite still being gold standard, are time consuming (Mokaddas and Ahmad, 2007). Molecular techniques are rapid, however these could accelerate the laboratory diagnosis of mycobacterial infection only when they combined with cultivation (Cheng *et al.*, 2005). In recent years, PCR based fingerprinting methods have been used for differentiation of NTM from *M. tuberculosis* complex and to identify NTM at the species level targeting many different genes (Mokaddas and Ahmad, 2007; Cheng *et al.*, 2005; Prammananan *et al.*, 2005; Chang *et al.*, 2002). The effort of Telenti *et al.* (1993) for designing a PCR based molecular typing method for differentiation of NTM to the species level is one of the most comprehensive technique which was based on amplification of the gene coding for the *hsp65*, followed by restriction enzyme analysis. The technique was proved to be able to differentiate the mycobacteria to species level based on their developed algorithm. The present study was undertaken with the aim of investigation of occurrence of NTM among patients refer to TB reference laboratory of Khuzestan Province, where there was no previous report on the isolation of these mycobacteria due to the lack of conventional identification procedures.

MATERIALS AND METHODS

A total of 150 samples were screened in this study for presence of NTM. The samples were obtained from patients referred to tuberculosis reference laboratory, PHLS of Khuzestan Province from June 2007 to March 2008. The proposal of this study was approved by human ethics committee at the university and the relevant authorities and we have had the committee's permission for access to patient demographic records. Besides, in spite of the culture was part of the essential diagnostic procedure for patients, informed consent has been taken from each referred patient. The samples were comprised of 84 sputum (56%), 52 urine (34.6%) and 14 others (bronchoalveolar lavage fluid, biopsy and gastric juice) (9.3%). The patients were 46 males and 54 females and their age ranged from 10 to 75 years with a mean of 36.24.

The samples were grown on Lowenstein Jensen (LJ) medium after initial decontamination and were kept at 37°C for 4-6 weeks. The isolated organisms were identified according to the standard biochemical identification tests including niacin accumulation, growth in LJ media containing either thiophen-carboxylic acid hydrazide (TCH), or ciprofloxacin; growth at 42 and 44°C; pigment production in light and dark, aryl sulphatase activity, catalase, nitrate reduction, hydrolysis of tween 80 (Winn *et al.*, 2007).

Template DNA was extracted from cultures by using the simple boiling method as previously described by Hosek *et al.* (2006). In brief, a few colonies grown on LJ medium were suspended in 0.5 mL deionized distilled water and was heated in a 100°C water bath for 15 min with subsequent centrifugation at 12000 rpm for 15 min. The supernatant was then used for amplification.

A 439 bp region of the 65 kDa *hsp* gene (Shinnick, 1987) was amplified with forward primer Tb11 (5'-ACCAACGATGGTGTGTCCAT- 3') and reverse primer Tb12 (5'-CTTGTCGAA CCGCATACCCT- 3') described by Telenti *et al.* (1993). The composition of PCR mixture was 50 mM KCl, 10 mM HCl (pH 8.3), 4.4 mM MgCl₂, 200 mM dNTPs, 0.5 µM of each primer,

2 U of *Taq* polymerase and 5 µL of DNA template in a final volume of 50 µL. All the reagents were purchased from Cinnagen Company, Tehran, Iran. The standard mycobacterial strains were obtained from Institute Pasteur, Iran and a non-mycobacterial strain was used as negative control.

The PCR conditions were initial denaturation of 95°C for 1 min followed by 35 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and then a final extension at 72°C for 10 min using a thermocycler (Techgene, UK). The PCR products were analyzed on 1% agarose gel. The gels were then stained with ethidium bromide and photographed.

The restriction enzymes *Bst*EII and *Hae*III (Fermentas, Germany) were used for the restriction fragment analysis of PCR products, as previously described by Telenti *et al.* (1993). Briefly, the amplicon was aliquoted into two tubes and digested with restriction endonucleases. Each digestion reaction mixture consisted of 10 µL of PCR product, 5 U of enzyme, 2.5 µL of enzyme corresponding buffer and water to a final volume of 25 µL. *Bst*EII and *Hae*III digestion were performed at 60 and 37°C, respectively for 60 min. The products were then separated on 2% agarose gel for REA analysis. The fragments were visualized by ethidium bromide staining and UV light. Gels were photographed, the digestion bands were measured. The base pair size of each DNA fragment was determined by comparison with the molecular marker.

RESULTS

In present study we analyzed the prevalence of the NTM species in patients referred to TB reference laboratory. The results from acid fast microscopy revealed that 37.3% of the smears were positive. The most prevalent smear positivity according to number of samples tested was seen in sputa (41 and 73.2%) which was comprised the majority of samples. Figure 1 represents the distribution of smear and culture positivity among the tested samples.

Out of 150 specimens subjected to culture during the study period, 90 (60%) were culture positive. Of these, two were contaminated and produced no visible band in amplification, so these were excluded from the study and the 88 rest were subjected to restriction analysis in the next step. Besides for comparing the conventional identification tests with molecular technique, the culture positives were identified by these tests and accordingly all 80 isolates (99.9%) were found to be *M. tuberculosis* on the basis of identification tests.

Based on the obtained results, 80 out of 88 clinical isolates (90.9%) subjected to PRA, showed the identical restriction patterns similar to *M. tuberculosis* reference strains of MTB H37Rv, equal to 160/145/72 bp fragments for *Hae* III and 250 /120/82 bp fragments for *Bst* EII digests (Table 1). The remaining eight isolates were identified as NTM and comprised of six isolates with restriction pattern

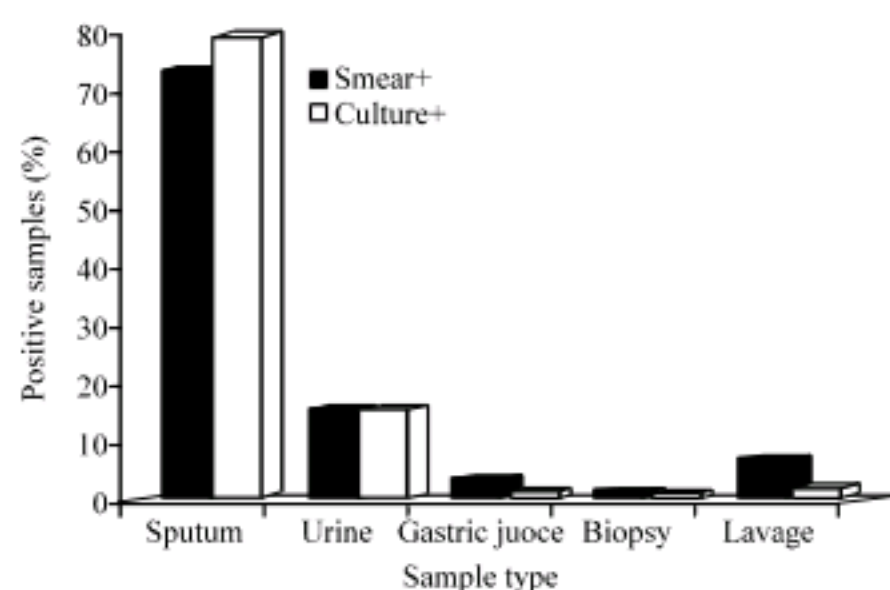


Fig. 1: Distribution of positive smear and culture in relation to type of samples tested

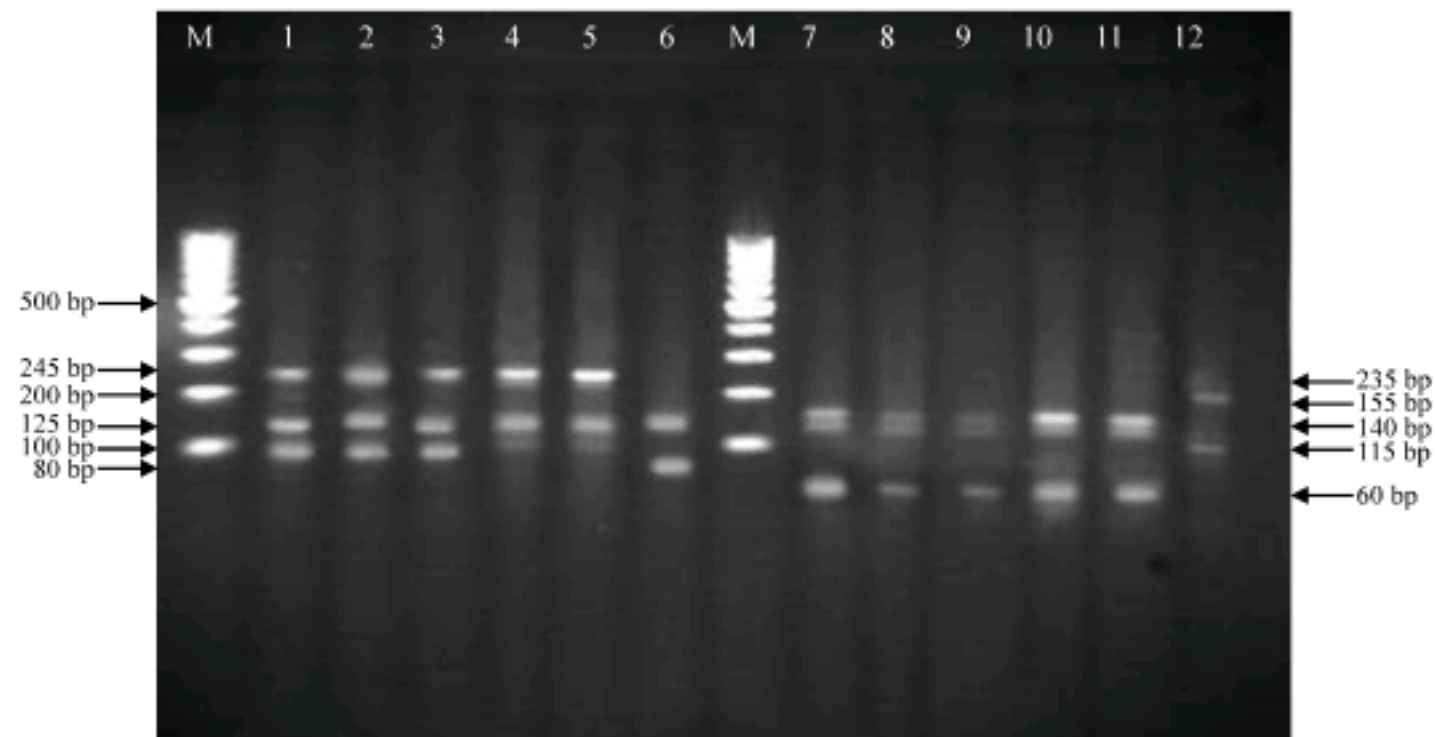


Fig. 2: Representative agarose gel of RFLP profiles for NTM isolates after digestion of PCR products with *Hae* III (lanes 7-12) and *Bst* EII (lanes 1-6) restriction enzymes. M: DNA size marker; Lanes 1-5 (*Hae* III 155/140/60 bp) and 7-11 (*Bst* EII 245/125/100 bp): *M. intracellulerae* isolates; lanes 6 (*Hae* III 235/115 bp) and 12 (*Bst* EII 125/80 bp): *M. gordonae* I isolate. *bp: Base pair

Table 1: Results from endonuclease restriction digest according to used algorithm

Identified <i>Mycobacterium</i> (No.)	Restriction endonuclease digested bonds (bp)	
	<i>Bst</i> EII	<i>Hae</i> III
<i>M. tuberculosis</i> H37Rv (1)	245/120/80	160/140/70
<i>M. tuberculosis</i> clinical strains (80)	245/120/80	160/140/70
<i>M. intracellulerae</i> (6)	245/125/100	155/140/60
<i>M. gordonae</i> I (2)	125/80	235/115

Table 2: Isolated mycobacteria on the basis of clinical samples

Type of ample	Identified mycobacteria	No. (%)
Sputum	<i>M. tubecculosis</i>	68 (77.27)
	<i>M. intracellulerae</i>	4 (4.54)
Urine	<i>M. tubecculosis</i>	8 (9.09)
	<i>M. intracellulerae</i>	2 (2.27)
	<i>M. gordonae</i> I	2 (2.27)
Others	<i>M. tuberculosis</i>	4 (4.54)
Total		88 (99.98)

compatible to *M. intracellulerae* and two isolates, showed restriction pattern equal to that reported for *M. gordonae* I (Fig. 2). Therefore, combining LJ medium culture and RFLP, 9.1% of the isolates were determined to be NTM.

The distribution of isolated mycobacteria in relation to original sample is presented in Table 2. While the rate of isolated NTM in sputum in total number of *M. tuberculosis* isolates was about 6%, in the urine samples the isolated NTM was consisted of half of the isolates recovered from urine. The majority of MTB isolates and all the NTMs were belonged to patients in age group of 41-60 years. No NTM was isolated from other minority samples included in present study.

DISCUSSION

The incidence of NTM infections has risen dramatically over the past decade, mainly due to increased in HIV infection. The isolation rate of NTM appears to vary considerably in different parts

of the world. No doubt, differences in transport of specimens and technique used are partly responsible but, obviously, there are variations in the prevalence of NTM. Besides, the predominant type of NTM also varies in different areas.

Distinction between tuberculosis and NTM disease based on clinical findings is very difficult. Conventional AFB staining and conventional culture alone are unable to differentiate *M. tuberculosis* from NTM strains (Koh *et al.*, 2005) and combining of these with biochemical tests require three to six weeks to complete (Chimara *et al.*, 2008).

In recent years, DNA based techniques have greatly facilitated identifying the species of NTM isolates. Among them, PCR- restriction enzyme analysis based on hsp 65 is a rapid and reliable method with broad application for identification of mycobacteria (Da Silva *et al.*, 2001; Park *et al.*, 2006; Martin *et al.*, 2007; Ryoo *et al.*, 2008).

In present study the prevalence of NTM in samples obtained from suspect tuberculosis patients was 9.1%. This prevalence was reported 13-25% in a study conducted in Czech Republic screening the isolates from 1999 to 2004, with *M. avium* complex as the most frequent NTM isolate (Polanecky *et al.*, 2006). In study of Bannalika and Verma (2006) the isolation of 22 mycobacteria were reported from 30 samples, of which three samples were identified as *M. avium* and the rest were *M. tuberculosis*. Naderi *et al.* (2006) were reported the prevalence of NTM in Iran Southeast as 33.3%. The rate of NTM in their study was higher than our study, probably due to the reason that their study was undertaken in a part of Iran with one of the highest rate of tuberculosis and other mycobacterial diseases.

From total samples under investigation in this study 60 samples (40%) were negative and failed to grow on LJ culture media and had a negative smear for AFB. Despite the advantages of PCR-RFLP technique, the only disadvantage of that is the failure of its application to sputa directly. Besides, as other investigators proposed, NTM might have failed to grow well on solid culture media and they grow better in liquid media (Kim *et al.*, 2008). These investigators have suggested to use duplex PCR- based method to complement traditional mycobacterial culture on solid media. Based on their explanation, we may have missed more NTM in this study, since liquid culture media are not routinely used in our TB center.

In this study, two contaminated isolates were not amplified with primers used. The reasons for lack of amplification were not identified, but the presence of PCR inhibitors could not be ruled out. Apart from these isolates failed to amplification, concordant results of RFLP and biochemical identification were obtained in all *M. tuberculosis* isolates compared to reference strain. Actually the technique was proved to be highly reliable for rapid identification of *M. tuberculosis* from culture and eliminates more weeks delay to have the results of biochemical tests. The earlier study showed that there was minor variation in *M. tuberculosis* strains using RFLP solely to evaluate the efficacy of technique for identification of *M. tuberculosis* isolates in various clinical samples (Khosravi and Hashemi, 2007).

None of our samples in this study was obtained from HIV positive patients according to the information recorded in TB centre archive. Since, NTM infections are predominant in AIDS patients as reported by Sampaio *et al.* (2001), Ferreira *et al.* (2002), Kang-Birken and Prichard (2006) and Nunes *et al.* (2008). This may explain the low rate isolation of these mycobacteria in present study.

In conclusion, RFLP is a valid alternative for phenotypic identification in the routine laboratory. It is fast, cheap and accurate for identification of pathogenic and potentially pathogenic mycobacteria.

ACKNOWLEDGMENTS

This study is financially supported by research affairs, Ahwaz Jondishapour University of Medical Sciences (M.Sc. Thesis with grant No. U-86116). We are indebted to Dr. A. Hashemi for providing standard strains of mycobacteria. Our thanks are due to Mrs. Eskandari from TB Reference Laboratory and other staff of laboratory for their help and collaboration.

REFERENCES

- Bannalikal, A.S. and R. Verma, 2006. Detection of *Mycobacterium avium* and *M. tuberculosis* from human sputum cultures by PCR-RFLP analysis of *hsp65* gene and *pncA* PCR. Indian J. Med. Res., 123: 165-172.
- Chang, C., L. Wang, C. Liao and S. Huang, 2002. Identification of nontuberculous mycobacteria existing in tap water by PCR-restriction fragment length polymorphism. Applied Environ. Microbiol., 68: 3159-3161.
- Cheng, V.C., W.W. Yew and K.Y. Yuen, 2005. Molecular diagnostics in tuberculosis. Eur. J. Clin. Microbiol. Infect. Dis., 24: 711-720.
- Chimara, E., L. Ferrazoli, S.Y.M. Ueki, M.C. Martins and A.M. Durham *et al.*, 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. Biomed. Cent. Microbiol., 8: 48-48.
- Da Silva, C.F., S.Y.M. Ueki, D.C.P. Geiger and S.C. Leao, 2001. *Hsp65* PCR-restriction enzyme analysis (PRA) for identification of mycobacteria in the clinical laboratory. Rev. Inst. Med. Trop. S. Paulo, 43: 25-28.
- Falkinham III, J.O., 1996. Epidemiology of infection by nontuberculous mycobacteria. Clin. Microbiol. Rev., 9: 177-215.
- Ferdinand, S., E. Legrand, K.S. Goh, M. Berchel and G. Mazzearelli *et al.*, 2004. Taxonomic and phylogenetic status of non-tuberculous mycobacteria in a Caribbean setting. Mol. Cell. Probes, 18: 399-408.
- Ferreira, R.M., M.H. Saad, M.G. Sliva and I.S. Fonseca, 2002. Non-tuberculous mycobacteria I: One year clinical isolates identification in tertiary hospital aids reference center, Rio de Janeiro, Brazil, in pre highly active antiretroviral therapy era. Mem. Inst. Oswaldo. Cruz., 97: 725-729.
- Frieden, T.R., T.R. Sterling, S.S. Munsiff, C.J. Watt and C. Dye, 2003. Tuberculosis. Lancet, 362: 887-899.
- Hernandez, M., G.P. Morlock, W. Raybutler, C. Crawford and R.C. Cooksey, 1999. Identification of *Mycobacterium* species by pcr-restriction fragment length polymorphism analyses using fluorescence capillary electrophoresis. J. Clin. Microbiol., 37: 3688-3692.
- Hosek, J., P. Svastova, M. Moravkova, I. Pavlik and M. Bartos, 2006. Methods of mycobacterial DNA isolation from different biological material: A review. Vet. Med., 51: 180-192.
- Jarzembowski, J.A. and M.B. Young, 2008. Nontuberculous mycobacterial infections. Arch. Pathol. Lab. Med., 132: 1333-1341.
- Johansen, I.S., 2006. Rapid diagnoses of mycobacterial diseases and their implication on clinical management. Doctor Med. Sci., 53: 28-45.
- Kang-Birken, S.L. and J.G. Prichard, 2006. *Mycobacterium szulgai* in a patient with advanced acquired immunodeficiency syndrome: An unusual pathogen with unusual multidrug resistance. Pharmacotherapy, 26: 1646-1649.
- Khosravi, A.D. and A. Hashemi, 2007. Application of restriction enzyme analysis technique based on 65kDa heat shock protein gene for fingerprinting and differentiation of *Mycobacterium tuberculosis* clinical strains isolated from tuberculosis patients in Ahwaz, Iran. Pak. J. Med. Sci., 23: 216-219.
- Kim, B.J., J.H. Park, S.A. Lee, H. Kim and C.Y. Cha *et al.*, 2008. Differentiation of mycobacteria in sputa by duplex polymerase chain reaction for mycobacterial *hsp65* gene. Diagnostic Microbiol. Infect. Dis., 62: 193-198.
- Koh, W.J., O.J. Kwon and K.S. Lee, 2005. Diagnosis and treatment of nontuberculous mycobacterial pulmonary diseases: A Korean perspective. J. Korean Med. Sci., 20: 913-925.

- Martin, A., C. Uwizye, K. Fissette, P. De Rijk, J.C. Palomino, S. Leao and F. Portaels, 2007. Application of the *hsp65* PRA method for the rapid identification of mycobacteria isolated from clinical samples in Belgium. J. Microbiol. Methods, 71: 39-43.
- Mokaddas, E. and S. Ahmad, 2007. Development and evaluation of a multiplex PCR for rapid detection of *Mycobacterium tuberculosis* complex members from non-tuberculous mycobacteria. Jap. J. Infect. Dis., 60: 140-144.
- Naderi, M., R. Alavi-Naini, B. Sharifi-Mood and M. Naserfar, 2006. Prevalence of tuberculosis and Non tuberculosis mycobacterium in Zahedan, Southeast of Iran. Res. J. Microbiol., 1: 375-377.
- Nunes, E.A., E.M. De Capitani, E. Coelho, A.C. Panunto, O.A. Joaquim and M.C. Ramos Mde, 2008. *Mycobacterium tuberculosis* and nontuberculous mycobacterial isolates among patients with recent HIV infection in Mozambique. J. Bras. Pneumol., 34: 822-828.
- Park, C.M., S.R. Heo, K.U. Park, J. Song, H.J. Lee, C.T. Lee and E.C. Kim, 2006. Isolation of nontuberculous mycobacteria using polymerase chain reaction-restriction fragment length polymorphism. Korean J. Lab. Med., 26: 161-167.
- Polanecky, V., P. Kalina, M. Kubin, B. Kozakova and M. Mullerova, 2006. Nontuberculous mycobacteria and incidence of mycobacterioses in Prague in 1999-2004. Epidemiol. Mikrobiol. Imunol., 55: 151-157.
- Prammananan, T., W. Cheunoy, P. Na-Ubol, N. Tingtoy, S. Srimuang and A. Chaiprasert, 2005. Evaluation of polymerase chain reaction and restriction enzyme analysis for routine identification of mycobacteria: Accuracy, rapidity and cost analysis. Southeast Asian J. Trop. Med. Public Health, 36: 1252-1260.
- Ryoo, S.W., S. Shin, M.S. Shim, Y.S. Park, W.J. Lew, S.N. Park, Y.K. Park and S. Kang, 2008. Spread of nontuberculous mycobacteria from 1993 to 2006 in Koreans. J. Clin. Lab. Anal., 22: 415-420.
- Sampaio, J.L., N. Artiles, R.M. Pereira, J.R. Souza and J.P. Leite, 2001. *Mycobacterium simiae* infection in a patient with acquired immunodeficiency syndrome. Braz. J. Infect. Dis., 5: 325-352.
- Shinnick, T.M., 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol., 169: 1080-1088.
- Telenti, A., F. Marchesi, M. Balz, F. Bally, E.C. Bottger and T. Bodmer, 1993. Rapid identification of *Mycobacteria* to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol., 31: 175-178.
- Winn, W., S. Allen, W. Janda, E. Koneman, G. Procop, P. Schreckenberger and G. Woods, 2006. Koneman's Color Atlas and Textbook of Diagnostic Microbiology. 6th Edn., Lippincott Williams and Wilkins, Philadelphia, USA.