# Comparison of Three Methods for the Identification of *Mycobacterium tuberculosis*

SADEGHIAN, A., TAVAKKOL AFSHARI J. [1]†, GHANAAT J., SADEGHAIN H., NAMAII MH AND KHODADOOST MA.†
*Microbiology laboratory, Ghaem Medical Center and †Immunology Research Group, Bu-Ali Research Institute, Mashhad University of Medical Sciences (MUMS), Mashhad-Iran*
[1]Corresponding author's e-mail: Jtavakkol@yahoo.com

## ABSTRACT

The present study was conducted to compare three methods, culture and morphology of colonies, biochemical tests and polymerase chain reaction for the identification of *Mycobacterium tuberculosis* by using well characterized clinical specimens from patients who were assessed according to standard parameters for *Mycobacterium tuberculosis*. Seventy seven isolated Mycobacterium species from patient's samples suspected to have tuberculosis from March 2003 till February 2004 collected for detection of *Mycobacterium tuberculosis* (MTB) or Mycobacterium other than tuberculosis (MOTT). After positive smear and cultures for *Mycobacterium tuberculosis* by standard methods, biochemical tests including catalase test, nitrate reduction, thiophene-2 carboxylic acid hydrazide (TCH) test and 68ºC catalase test were performed. For Polymerase Chain Reaction (PCR) we used the commercial Cinagene® PCR detection kit for *Mycobacterium tuberculosis*. The results for biochemical tests indicated that, 49(63.6%) of isolated colonies, detected as MTB and 28(36.4%) detected as MOTT. Results from the PCR method indicated that 51(66.2%) of isolated colonies, identified as MTB and 26(33.8%) detected as MOTT. Morphological characteristic of isolated colonies identified 54(70.1%) as having rough appearance, a characteristic for MTB and 23(29.9%) as having smooth appearance, a characteristic for MOTT. Our results indicated that the sensitivity and specificity of biochemical tests compared to PCR method were 96 and 100%, respectively. Sensitivity and specificity of morphological characteristic for isolated colonies in comparison with PCR method were 98% and 84% respectively. In this study, there were no statistically significant differences between biochemical tests, morphology of colonies and PCR method.

**Key Words:** Mycobacterium; Tuberculosis; PCR; Identification; Culture

## INTRODUCTION

The diseases produced by species of the genus Mycobacterium are important causes of morbidity and mortality in the world, particularly in third world countries; they have increased due to HIV infections, with the involvement mainly of *M. tuberculosis* and *M. avium* complexes. A recent report suggests that there are 20 million cases of active tuberculosis in the world and that around 5000 people die from tuberculosis every day (Barnes *et al.*, 1991). The annual incidence of the disease is expected to be increased by 41% between 1998 to 2020 and achievement of WHO targets by 2010 would prevent 23% cases by 2020 (Dye *et al.,* 1999). The identification of Mycobacterium to the species level is important because of the clinical significance; some species are pathogenic while others are not (Kamerbeek *et al.*, 1997). Knowledge of species is also critical in order to provide adequate patient management because; specific antimycobacterial drugs are required against different pathogenic Mycobacterium species (Gamboa *et al.*, 1998). The conventional methods for the identification of Mycobacterium currently are based on culture and biochemical tests. However because the organism is slowly growing, laboratory diagnosis by these methods can take as along as eight to ten weeks (Springer *et*

*al.*, 1996). Alternative techniques have been established, such as thin layer chromatography, gas-liquid chromatography, high-performance liquid chromatography (HPLC), and molecular techniques based on amplification, or sequencing of nucleic acids (Eisenach *et al.*, 1991; Cormican *et al.*, 1992; Kirschner *et al.*, 1993; Fodor, 1995).

This study was designed to compare three methods; culture and morphology of colonies, biochemical tests and polymerase chain reaction for the identification of *M. tuberculosis* by using well characterized clinical specimens from patients who were assessed according to standard parameters for *M. tuberculosis*.

## MATERIALS AND METHODS

In a prospective study from March 2003 till February 2004 a total of seventy seven isolated Mycobacterium species from various specimens of patients who were suspected of having tuberculosis at Ghaem medical center in Mashad-Iran were collected for detection of *Mycobacterium tuberculosis* (MTB) or Mycobacterium other than tuberculosis (MOTT) by classical culture, biochemical tests and PCR method. The type of specimens included sputum, bronchial washing and pleural fluid. Specimens were processed by standard mycobacterium laboratory method.

In this study, Petroff's method was used to process and decontaminate clinical specimens (Bartelt, 2000). Briefly, specimens were mixed by the same volume of 2% NaOH and 1% NaCl. After 15 minutes incubation, phosphate buffer was added and centrifuged at 3000 *g* for 15 minutes. Then the supernatant was withdrawn and the sediment was neutralized by mixture of 1M HCl and phenol red (as indicator) and was used for staining and culture.

For staining, 200 µl of samples were placed on the surface of slides and after drying, they were fixed in 80ºC for 30 minutes. Ziehl-Neelsen (Smithwick, 1976) method was used for acid fast staining.

Culture of Mycobacterium was performed by inoculation of the processed material onto Lowenstein-Jenson (Baron *et al.*, 1990) and incubated at 37ºC for six weeks.

Positive cultures were further tested by biochemical tests and PCR method. Also growth rate and pigment production were determined for each isolate. Biochemical tests performed for each isolate were nitrate reduction, semi-quantitative catalase test, catalase production at 68ºC, and thiophene-2 carboxylic acid hydrazide (TCH) test (Baron *et al.*, 1990).

For PCR we used the commercial Cinagene® PCR detection kit for *Mycobacterium tuberculosis* (Tehran, Iran). Basically, bacterial cells were lysed with TB lyses solution included with the kit; DNA was then extracted by boiling method (Holmes *et al.*, 1981). Reaction conditions included a pre-incubation time of 2 min at 93 ºC, followed by 40 two-step cycles of 20 seconds at 93ºC, and 30 seconds at 72ºC. PCR products were electrophoresed on 2% agarose gels.

Result data were entered into a database, to calculate the sensitivity for each method and concordance percentages between methods.

## RESULTS

Data from biochemical tests are shown in Table I. These results indicated that, 49(63.6%) of isolated colonies, identified as MTB and 28(36.4%) detected as MOTT. Results by PCR method showed that 51(66.2%) of isolated colonies were identified as MTB and 26(33.8%) as MOTT (Fig.1-2). The morphological characteristic of isolated colonies identified 54(70.1%) as having rough appearance, a characteristic for MTB and 23(29.9%) as having smooth appearance, a characteristic for MOTT. Our results indicated that the sensitivity and specificity of biochemical tests compared to PCR method were 96 and 100%, respectively. Positive predicted value (PPV) and negative predicted value (NPV) were 100 and 92%, respectively (Table II, Fig. 3). Sensitivity and specificity of morphological characteristic for isolated colonies in comparison with PCR were 98 and 84% and its PPV and NPV were 92 and 95%, respectively (Table II, Fig. 4). Overall results indicated that there were no statistically

**Fig. 1. PCR amplification for *M. tuberculosis*. Lane1: *M. tuberculosis* positive control; Lanes 2-5 and 7: *M. tuberculosis* positive specimens; Lanes 8-10: *M. tuberculosis* negative specimens, lane 6: 50 bp ladder.**
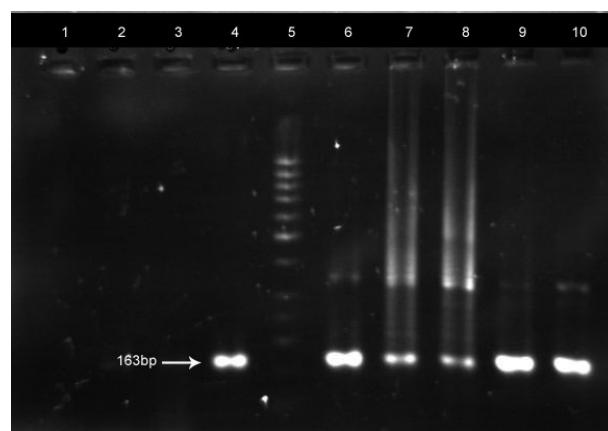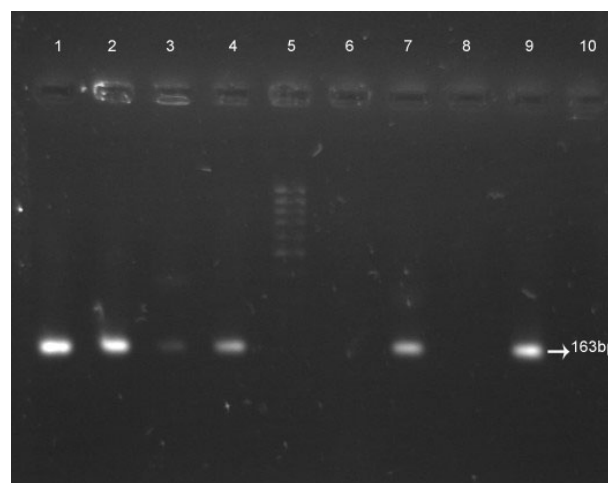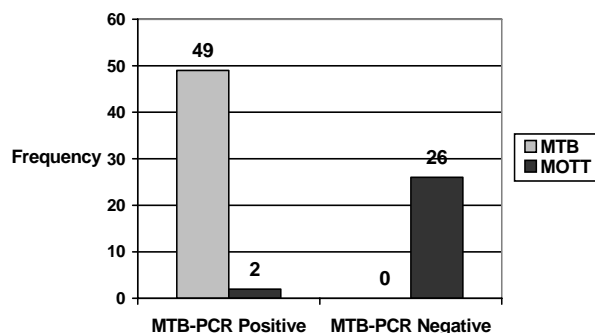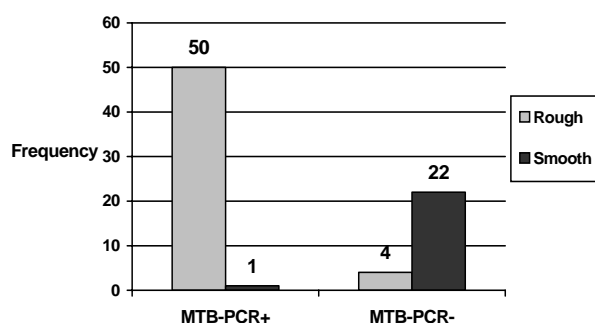


**Fig. 2. PCR amplification for *M. tuberculosis*. Lane1: *M. tuberculosis* positive control; Lanes 2, 4, 7, and 9: *M. tuberculosis* positive specimens; Lanes 3, 6, and 8: *M. tuberculosis* negative specimens; Lane 5: 50 bp ladder.**



significant differences between biochemical tests, morphology of colonies and PCR method.

## DISCUSSION

The ability to rapidly detect *M. tuberculosis* in clinical specimens has important implications in the treatment of TB (Wang & Tay, 1991). In Iran, laboratory diagnosis of TB is mostly based on smear-microscopy, classical culture and biochemical identification methods. Classical culture and biochemical tests, when properly applied, detect *M. tuberculosis* in clinical samples, with reasonable sensitivity (Morgan *et al.*, 1983; Mondragon *et al.*, 2000; Iqbal *et al.*, 2003). However, primarily due to the slow growth of the

**Fig. 3. Comparison of PCR results with biochemical tests for detection of Mycobacterium species.**



**Fig. 4. Comparison of PCR results with morphology of colonies for detection of Mycobacterium species.**



bacteria, these methods usually require 4 to 8 weeks for completion. This results in numerous missed or delayed diagnoses, adversely affecting patient care and tuberculosis control and allows for the spread of infection (Bennedsen *et al.*, 1996). Today, many molecular techniques are available for the diagnosis of tuberculosis. These techniques are fast, reliable and sensitive (Ellner *et al.*, 1988; Cousins *et al.*, 1992; Devallois *et al.*, 1997). However some studies indicated a relatively low PCR sensitivity. Pierre (1991) and Soini (1992) found sensitivity for their PCR assays of 63 and 55.9%, respectively. Such low sensitivity in PCR may be explained by suboptimal assay conditions. As demonstrated by Cegielski (1997), the sensitivity of PCR with pericardial fluid for detection of MTB was poor in compare to conventional culture method. In their study tuberculosis was correctly diagnosed by culture in 15 (93%) patients, by PCR in 13 (81%) patients, and by histology in 13 of 15 (87%) patients. Also, PCR gave one false-positive result for a patient. Therefore, the sensitivity and false-positive results with PCR remain a concern.

On the other hand, other studies showed that PCR assays not only can prove more sensitivity than conventional culture method, but can modify the present understanding of MTB infection (Del Portillo *et al.*, 1991;

Grosset & Mouton, 1995). In few countries, nucleic acid amplification techniques are used mainly for cases where there is a chance that the infection may be due to a mycobacterium other than M. tuberculosis (Hance *et al.*, 1988). It is also to be noted that PCR techniques involve prohibitive expenditure in terms of instrumentation, expertise and reagents, putting them out of reach of many laboratories in developing countries including Iran.

In our study, seventy seven isolated colonies from positive Mycobacterium cultures were differentiated into MTB and MOTB based on three different methods. Results obtained in this work showed that the basic methodology for Mycobacterium identification, including colonial morphology, pigmentation, growth rate, and enzymatic tests were fast and reliable for the identification of MTB isolates. The overall accuracy of PCR approached the results of conventional methods. These results indicate that, for diagnosis of MTB from positive colonies, conventional biochemical tests are sensitive as the PCR technique. Studies carried out in developed countries have demonstrated similar sensitivities for the new methods and conventional culture (Sjobring *et al.*, 1990; Noordhock *et al.*, 1994). Several studies evaluated commercial amplification kits designed to directly detect MTB DNA and RNA in clinical specimens (Morgan *et al.*, 1983; Piersimoni *et al.*,

**Table I. Results of biochemical tests for detection of MTB**

| Test | Result | Frequency | Percent | Cumulative Percent |
|---|---|---|---|---|
| Catalase test | >45 mm | 18 | 23.4 | 23.4 |
| | <45 mm | 59 | 76.6 | 100.0 |
| | Total | 77 | 100.0 | |
| Nitrate reduction test | Negative | 16 | 20.8 | 20.8 |
| | + | 18 | 23.3 | 44.1 |
| | ++ | 13 | 16.9 | 61.0 |
| | +++ | 30 | 39 | 100.0 |
| | Total | 77 | 100.0 | |
| TCH test | Resistance (Negative) | 76 | 98.7 | 98.7 |
| | Sensitive (Positive) | 1 | 1.3 | 100.0 |
| | Total | 77 | 100.0 | |
| Catalase 68$^{oc}$ test | Negative | 54 | 70.1 | 70.1 |
| | Positive | 23 | 29.9 | 100.0 |
| | Total | 77 | 100.0 | |

**Table II. Comparison of biochemical tests and Morphology results with PCR for detection of MTB**

| | | | Total | Biochemical Tests' Results | | Morphology of Colonies | |
|---|---|---|---|---|---|---|---|
| | | | | MTB | MOTT | Smooth | Rough |
| PCR Results for detection of MTB | Positive | Frequency | 51 | 49 | 2 | 1 | 50 |
| | | Percent | 66.2% | 96.1% | 3.9% | 2.0% | 98.0% |
| | Negative | Frequency | 26 | 0 | 26 | 22 | 4 |
| | | Percent | 33.8% | 0.0% | 100.0% | 84.6% | 15.4% |
| Total | | Frequency | 77 | 49 | 28 | 23 | 54 |
| | | Percent | 100.0% | 63.6% | 36.4% | 29.9% | 70.1% |

1997; Scarparo *et al.*, 2000). The amplification techniques had a strong impact on the speed of the TB diagnosis. However, these studies indicate that, PCR cannot replace Mycobacterium culture.

## CONCLUSIONS

If we consider PCR as the most sensitive and specific method for the detection of MTB, then, our results indicated that, biochemical tests have a good correlation with PCR for this purpose, although, as far as cost-benefit analysis, biochemical tests are less expensive than PCR. Even the morphology of colonies in culture, has close sensitivity and specificity to PCR. Due to the expensive equipments and reagents to perform PCR method, we suggest that, for suspected specimens for MTB, standard culture and biochemical tests are sufficient, if time is not critical.

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