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Full Article

The Comparison of Biochemical and Sequencing 16S rDNA Gene Methods to Identify Nontuberculous *Mycobacteria*

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ABSTRACT

The identification of *Mycobacteria* in the species level has great medical importance. Biochemical tests are laborious and time-consuming, so new techniques could be used to identify the species. This research aimed to the comparison of biochemical and sequencing 16S rDNA gene methods to identify nontuberculous *Mycobacteria* in patients suspected to tuberculosis in Golestan province which is the most prevalent region of tuberculosis in Iran. Among 3336 patients suspected to tuberculosis referred to hospitals and health care centres in Golestan province during 2010-2011, 319 (9.56%) culture positive cases were collected. Identification of species by using biochemical tests was done. On the samples recognized as nontuberculous *Mycobacteria*, after DNA extraction by boiling, 16S rDNA PCR was done and their sequencing were identified by NCBI BLAST. Of the 319 positive samples in Golestan Province, 300 cases were *M.tuberculosis* and 19 cases (5.01%) were identified as nontuberculous *Mycobacteria* by biochemical tests. 15 out of 19 nontuberculous *Mycobacteria* were identified by PCR and sequencing method as similar by biochemical methods (similarity rate: 78.9%). But after PCR, 1 case known as *M.simiae* by biochemical test was identified as *M. lentiflavum* and 3 other cases were identified as *Nocardia*. Biochemical methods corresponded to the 16S rDNA PCR and sequencing in 78.9% of cases. However, in identification *of M. lentiflavum* and Nocaria sp. the molecular method is better than biochemical methods.

Keywords: Nontuberculous *Mycobacteria*, 16S rDNA, *M. simiae*, *M. lentiflavum*

INTRODUCTION

Mycobacteria belong to Mycobacteriacea family and Actinomycetale order (Palomino *et al* 2007). All species of *Mycobacteria* other than members of *Mycobacterium tuberculosis* complex called Nontuberculous *Mycobacteria* or atypical *Mycobacteria* which exist in water and soil around the world (Dieudonne 2012). Nontuberculous *Mycobacteria* are sometimes known as human pathogen and opportunistic pathogens. They often

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cause pulmonary diseases and they are transferred by inhalation of aerosols. However, so many cases of skin, lymphadenitis and diffused diseases have been reported in recent years (Simons *et al* 2011). In many laboratories only the direct smear or colony appearance in Lowenstein media is used for diagnosis of tuberculosis. However, these methods are not able to identify *Mycobacteria* at the species level, so sometimes anti-tuberculosis treatment mistakenly are used for a variety of nontuberculous *Mycobacteria* and therefore, failure in the tuberculosis treatment is observed. So, determining *Mycobacteria* species to reach accurate recognition of tuberculosis species and its discrimination from different kinds of nontuberculous *Mycobacteria* should be performed in professional laboratories (Greco *et al* 2009). For detection of *Mycobacteria*, there are different ways. Biochemical tests after the culturing are very slow and time consuming, and common procedures such as niacin and TCH are not suitable to identify different nontuberculous *Mycobacteria* and even different strains of *M. tuberculosis* (Richardson *et al* 2009). PCR is a useful technique for detection and identification of Mycobacterial infections (Haldar *et al* 2009). This method is simple, rapid, reliable and repeatable, which can often be used to suspect the medical and veterinary laboratories (Moure *et al* 2011). Review of several gene polymorphisms in specific areas, such as the 32-kDa protein coding genes, genes dnaJ, gene sod, gene rpoB, the gene hsp65, gene (16S rDNA), rrs and IS (especially IS6110 used for detection of *Mycobacterium tuberculosis*) can provide quick and easy identification of *Mycobacteria* species from each other. In the 1980s, researchers demonstrated that the phylogenetic relationship between bacteria and their lifestyle can be compared through a stable genetic code. The genetic code candidate for bacteria can be the coding gene 16S rDNA. Segment of DNA that now commonly is used for classification of bacteria, which sometimes is called 16S rDNA (Howell *et al* 1997). This study was performed in order to compare the performance of two methods based on classical biochemical tests and other methods of sequencing of the 16S rDNA PCR for identification of nontuberculous *Mycobacteria*.

MATERIALS AND METHODS

Sample collection. This descriptive study was carried on 3336 patients suspected to tuberculosis who referred to hospitals and Health care centers of Golestan province for one year, from 2010 –11, and 545 cases (16.33%) in Acid fast staining had positive smear and 319 (9.6%) positive cultures of *Mycobacteria* were isolated to be studied in this research. These samples were collected from among 275 pulmonary and 44 extra pulmonary tuberculosis patients including: Bronchoalveolar lavage "BAL" (27 cases), Gastric lavage (8 cases), Abscess (5 cases), pleural samples (3 cases), CSF (1 case). Informed consent was obtained from each patient included in the study,and patient information remains confidential.

Biochemical method for species Identification. In order to determine species, we used biochemical tests including: the growth rate, morphology of colony, production of pigment, nitrate reduction, presence of pyruvate, niacin test, semi quantitative catalase, 68^{o}c catalase, tween hydrolysis, arylsulfatase, growth on macconkey agar without crystal violet, tellurite reduction, tolerance to the NaCl %5, urease and Iron uptake tests (Forbes *et al* 2007). For samples identified as nontuberculous *Mycobacteria* in biochemical method, the molecular techniques were performed as follows:

DNA extraction. For samples identified as nontuberculous *Mycobacteria* in biochemical tests, the DNA extraction was prepared by boiling method. For this purpose, 2-3 colonies were inoculated in 100 µl of sterile distilled water and then 100 µl of chloroform was added, the suspension was boiled in 80 ^{o}c for 20 minutes, then it was centrifuged for 1 min in 12000 cycles. The supernatant was separated and the presence of DNA was tested by electrophoresis on 1% gel Agarose (Williams *et al* 2007).

16S rDNA PCR. The primers were selected according to Han et al (Han *et al* 2002) study and after blast in NCBI software, they were used for amplification of 16S rDNA tested Mycobacteria as follow:
(Forward) 5'-GCGTGCTTAACACATGCAAGTC-3' and (Revers) 5'-TCCTCCTGATATCTGCGCATTC-3' (Generay Biotech Co.). PCR reaction master mix in 50 µl volume included 5 µl of buffer, 5 µl of MgCl2, 0.25 µl of dNTP, 0.5 µl of Taq polymerase, 1 µl of each primer, 5 µl of extracted DNA and 33.25 µl of water were obtained (Han *et al* 2002). PCR protocol was as follow; 95 ^{o}c for 5 minutes, 40 cycles include 94 ^{o}c for 20 seconds, 55 ^{o}c for 20 seconds and 72 ^{o}c for 40 seconds. The process of DNA amplification was performed in Ependrof Thermocycler and it was held 2 minutes in 8 ^{o}c. PCR product was electrophoresed on 1.5 % gel agarose. Created band width was variable from 620 bp to 740 bp. For all cases that band was observed, sequencing of product was performed by Macro gene Co. After recognizing the sequence, chromas software used for sequencing analysis and the species were identified by NCBI BLAST. Data was entered in SPSS 16 software and it was analyzed by X^2 test.

Table 1. Percent of similarity in Allignment

Number	Species	Similarity
1	*M. simiae*	99%
2	*Nocardia*	99%
3	*M. simiae*	99%
4	*M. chelonae*	93%
5	*M. fortuitum*	99%
6	*M. lentiflavum*	99%
7	*Nocardia*	99%
8	*M. gastri*	99%
9	*M. nonchromogenicum*	96%
10	*M. simiae*	99%
11	*M. fortuitum*	99%
12	*M. gordonae*	98%
13	*M. simiae*	99%
14	*M. fortuitum*	99%
15	*M. flavescens*	96%
16	*M. fortuitum*	99%
17	*Nocardia*	99%
18	*M. simiae*	99%
19	*M. simiae*	99%

RESULTS

Based on biochemical methods on 19 cases, 7 isolates were identified which *M. simiae* (7 cases) and *M. fortuitum* (4 cases) were the most common species; and from each of these groups, 1 case was observed: *M.chelonae*, *M.flavescens*, *M.gastri*, *M.gordonae* and *M.nonchromogenicum*. By this technique, we were not able to diagnose the 3 cases including 2 rapid grown and one scotochromogenic *Mycobacteria*. 16S rDNA sequencing technique confirms biochemical finding in 15 cases (78.9%). On the other hand, from 19 cases, which were identified as nontuberculous *Mycobacteria* by biochemical tests, 4 cases had controversial result with molecular method. One case which identified as *M. simiae* by biochemical method, but by using PCR method, it was proved to be *M. lentiflavum*. In the last three cases we were not able to identify the species of *Mycobacteria* by biochemical procedures but they diagnosed as *Nocardia* sp by sequencing of 16S rDNA method (Figure 1).

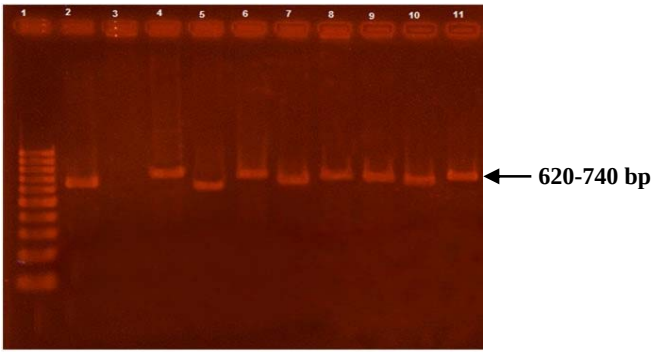

Figure 1. electrophoresis of PCR product 16S rDNA gene of nontuberculous *Mycobacteria* isolated from patients in Gorgan. Line 1: DNA Ladder (100bp), 2: positive control (*M. tuberculosis*), 3: negative control, 4: *M. nonchromogenicum* (736 bp), 5: *M. chelonae* (622 bp), 6*: M. fortuitum* (720), 7: *M. gastri* (642 bp), 8: *Nocardia* (705 bp), 9: *M. flavescens*: (697 bp), 910: *M. gordonae*(645 bp), 11: *M. simia* (706bp).

DISCUSSION

The findings of this study indicated that PCR 16S rDNA gene and standard biochemical tests confirms each other on the diagnosis of nontuberculous *Mycobacteria* but the *Nocaria* species were not properly identified by the biochemical tests applied in our study. *Nocardia* is acid-fast (partial Acid fast), rod like bacteria which can grow in Lowenstein Jensen medium and it may be easily mistaken with *Mycobacteria* species because both are in the Order of Actinomycetales and the Suborder of Corynebacterineae

Table 2. The identification of nontuberculous *Mycobacteria* based on biochemical test and its comparison with 16S rDNA sequencing test

number	Molecular detection	Biochemical detection	growth rate	Morphology	Pigment	Niacin	Catalase (nm)	Nitrate	68°c catalase	tween hydrolysis	tellurite reduction	growth on Macconkeyagar without crystal violet	urease	tolerance to the NaCl %5	Arylsulfatase	Iron uptake
1	*M.simiae*	*M.simiae*	Slow	Smooth	-	+	120	-	+	-	+	-	-	-	-	-
2	*Nocardia*	unrecognizable Fast-growing *Mycobacteria*	Rapid	Smooth	-	-	50	+	-	+	+	+	+	+	-	+
3	*M.simiae*	*M.simiae*	Slow	Smooth	-	+	73	-	+	-	+	-	-	-	-	-
4	*M.chelonae*	*M.chelonae*	Rapid	Smooth	-	-	120	-	+	-	+	+	+	-	+	-
5	*M.fortuitum*	*M.fortuitum*	Rapid	Smooth	-	-	50	-	+	+	+	+	+	+	+	+
6	*M.lentiflavum*	*M.simiae*	Slow	Smooth	+	-	120	-	+	-	+	-	-	-	-	-
7	*Nocardia*	Unrecognizable scotochromogen	Rapid	Smooth	+	-	90	-	+	-	-	-	+	-	-	-
8	*M.gastri*	*M.gastri*	Slow	Smooth	-	-	120	-	+	+	+	-	+	-	-	-
9	*M.nonchromogeni-cum*	*M.nonchromoge-nicum*	Slow	Smooth	-	-	50	+	+	+	-	-	-	-	-	-
10	*M.simiae*	*M.simiae*	Slow	Smooth	-	+	70	-	+	-	+	-	-	-	-	-
11	*M.fortuitum*	*M.fortuitum*	Rapid	Smooth	-	-	50	-	+	+	+	+	+	+	+	+
number	**Molecular detection**	**Biochemical detection**	**growth rate**	**Morphology**	**Pigment**	**Niacin**	**Catalase (nm)**	**nitrate**	**68°c catalase**	**tween hydrolysis**	**tellurite reduction**	**growth on Macconkey agar without crystal violet**	**urease**	**tolerance to the NaCl %5**	**Arylsulfatase**	**Iron uptake**
12	*M. gordonae*	*M. gordonae*	Slow	Smooth	-	-	55	+	+	+	-	-	+	-	+	-
13	*M. simiae*	*M. simiae*	Slow	Smooth	-	+	75	-	+	-	+	-	-	-	-	-
14	*M. fortuitum*	*M. fortuitum*	Rapid	Smooth	-	-	80	-	+	+	+	+	+	+	+	+
15	*M. flavescens*	*M. flavescens*	Slow	Smooth	+	-	50	+	+	+	+	-	+	+	-	-
16	*M. fortuitum*	*M. fortuitum*	Rapid	Smooth	-	-	160	-	+	+	+	+	+	+	+	+
17	*Nocardia*	unrecognizable Fast-growing *Mycobacteria*	Rapid	Smooth	-	-	50	+	-	+	+	+	+	+	-	+
18	*M. simiae*	*M. simiae*	Slow	Smooth	-	+	74	-	+	-	+	-	-	-	-	-
19	*M. simiae*	*M. simiae*	Slow	Smooth	-	+	75	-	+	-	+	-	-	-	-	-

(Nour *et al* 2011). On the other hand, the biochemical and molecular techniques have average harmony in diagnosis of nontuberculous *Mycobacteria*; however, biochemical methods are time consuming, requiring several tests and having the possibility of contamination of the environment; therefore, it seems that the molecular methods particularly sequencing PCR product of 16S rDNA gene can be substituted for identification of nontuberculous *Mycobacteria*. In the study conducted by Shojaei and et al., 8 independent strains characterized rapid growth and scotochromogenic colonies were isolated from 6 different countries of the world. DNA based analysis revealed unique sequence in the 16S rRNA gene, the rRNA gene internal transcribed spacer 1, genes encoding the 65 KDa heat shock protein, beta-subunit of RNA polymerase. Consequently, the genotypic and phenotypic data both strongly supported the inclusion of the strains as members of a novel species named *M. iranicum* sp. nov. (Shojaei *et al* 2013). In USA, Han et al which worked on 69 cases of acid fast *Mycobacteria* identified by using biochemical tests and 16S rDNA gene PCR, only 8 cases (11.6%) of 16S rDNA gene PCR and biochemical test were not similar, and in 1 case *M. lentiflavum* was wrongly diagnosed as *M. simiae* by biochemical tests (Han *et al* 2002). In another study by Terese et al. in at Chennai in India working on 47 samples using biochemical tests and 16S rDNA gene PCR in 33 cases (70.2%), biochemical tests were confirmed by PCR (Therese *et al* 2009). In the study which performed by Yam et al in Hong Kong, a total of 1167 samples using biochemical tests was not able to identify 10 cases including 3 cases scotochromogen, 1 case photochromogen, 2 cases nonchromogen and 4 cases fast growing *Mycobacteria* but they were diagnosed by 16S rDNA gene PCR (Yam *et al* 2006). Our findings are similar to the data of earlier studies which is suggesting that molecular methods 16S rDNA gene PCR can be used to substitute the biochemical method in identifying nontuberculous *Mycobacteria*. Our study is similar to other ones which showed that in the identification of *M. lentiflavum* biochemical tests proved not to be a decisive procedure and the use of molecular method 16S rRNA gene PCR is strongly suggested. *M.lentiflavum* was introduced in 1996, slow growing scotochromogenic bacteria, which is similar to *M. avium* and *M. simiae* and their differentiation based on biochemical method is difficult (Marshall *et al* 2011). This later issue emphasized once again on the importance and significance of molecular techniques for the identification of nontuberculous *Mycobacteria* instead of biochemical procedures.

Ethics

Since the bacterial colony was tested and the patient's name was not mentioned, this study doesn't face any legal problem. In addition, patients were voluntarily tested.

Conflict of Interests

There is no conflict between the authors of this manuscript.

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