Evaluation of Duplex-PCR Based Method to Differentiate *Mycobacterium Tuberculosis* Complex and *Non-Tuberculous Mycobacteria* Using RNA Polymerase (*Rpob*) Gene

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Abstract: The detection of *Mycobacterium tuberculosis* (*M.tuberculosis*) in clinical samples is a major priority in the control of tuberculosis. Since the early 1980s, there has been an increase in diseases caused by *Non-tuberculous Mycobacteria* (NTM), affecting immunocompetent and immunocompromised persons. Mixed infections have been reported. Therefore, it is important to be able to differentiate both organisms during the early stage of diagnosis. Identification by conventional biochemical tests have been fraught with a long turn-around time, other methods based on lipid analysis are used only in few laboratories. The PCR linked methods using specific target genes have provided alternative rapid approaches. This study aimed at the evaluation of the efficiency and usefulness of duplex-PCR (DPCR) targeting RNA polymerase (*rpoB*) gene as a tool to differentiate *M. tuberculosis* complex from NTM strains from suspected patients of pulmonary tuberculosis compared to conventional culture-based methods. Forty one mycobacterial strains, isolated from sputum samples of pulmonary tuberculosis suspected patients, were subjected to different phenotypic (conventional culture and biochemical tests) and genotypic methods (DPCR targeting *rpoB* gene), for identification and differentiation of *M.tuberculosis* complex and NTM isolates. Sequencing and restriction fragment length polymorphism (RFLP) were used to assist in the further identification of NTM strains. Thirty seven isolates identified as *M.tuberculosis* phenotypically, produced an amplicon of 235 bp using DPCR, while the remaining 4 isolates identified as NTM by phenotypic methods, produced an amplicon of 136 bp by the same assay. Accordingly, DPCR assay revealed 100% sensitivity and specificity as compared to phenotypic tests. Moreover, the four 136 bp amplicons when analyzed by sequencing and RFLP using *Hae III* and *Msp I* restriction enzymes, showed complete concordance with the phenotypic results. DPCR assay based on *rpoB* gene with 2 different sets of primers amplifying 2 different sized DNA from a single target gene provides a rapid and reliable mean for the differential identification of *M.tuberculosis* and NTM isolates in cultures, even coexistence of both organisms could be detected by the presence of 2 different amplicons in a single reaction mixture. Further studies are needed to evaluate other target genes in comparison with our gene, and to determine the applicability of these molecular assays on clinical samples.

[Abdel Aziz HA, El Hefnawy AM, Harfoush RA, El Sheikh RS. **Evaluation of Duplex-PCR Based Method to Differentiate *Mycobacterium Tuberculosis* Complex and *Non-Tuberculous Mycobacteria* Using RNA Polymerase (*Rpob*) Gene.** *Biomedicine and Nursing* 2018;4(2): 72-78]. ISSN 2379-8211 (print); ISSN 2379-8203 (online). http://www.nbmedicine.org. 13. doi:10.7537/marsbnj040218.13.

Key words: Mycobacteria, RFLP, Sequencing.

1. Introduction

Tuberculosis (TB) has been treated effectively for many years. Nevertheless, it is still the second leading cause of death from an infectious disease worldwide (WHO, 2006).

According to the WHO, there were an estimated 9.4 million incident cases of TB globally in 2008. Most of these cases occurred in Asia and Africa, with small proportions in the Eastern Mediterranean region, the European region and the Americas. The twenty two high –burden countries account for 80% of all estimated cases worldwide. (WHO, 2009 'a') Although Egypt is not one of this WHO's list, it is considered one of the high incidence countries in the Eastern Mediterranean region, and TB is considered the second most important public health problem in Egypt. (WHO, 2009 'b')

The proportion of nontuberculous mycobacteria (NTM) increased globally during the period 2000 - 2008 from 32.3% to 49.8%. *M. avium* complex and *M.abcessus* were the most frequently isolated. (Lai et al., 2010). There is a marked geographic variability worldwide in the prevalence rates of Pulmonary NTM. (Marras and Daley, 2002)

Understanding of TB epidemiology and the efficacy of control activities have been complicated by the emergence of drug-resistant bacilli and by the synergism of TB with HIV coinfection. (Corbett et al., 2003).

NTM infection can cause clinical problems, as its pathogenic potential and susceptibilities to antituberculous treatments vary. (Wolinsky, 1992) In addition, mixed infections of (*M.tuberculosis*) and NTM have been reported. (Libanore et al., 1992)

Therefore, it has become important to be able to differentiate between the two during the early stage of the diagnostic procedure.

The conventional biochemical tests available for *Mycobacterium* species identification have proven to be difficult to use, time consuming and often giving incorrect identification. (Rothschild et al., 2001; Kaotch, 2004; Tsang, 1983). In addition, with the recent developments in molecular techniques and the availability of genome sequencing data, several molecular tests have been developed. (Rogall, 1990) The *IS6110* element (Yuen et al., 1993), the*16S rRNA* gene, the *hsp65* gene, the intergenic region between 16S and 23rRNA (Lee et al., 2003) the *mtp40* gene (Yuen et al., 1993) and the *rpoB* gene are among the targets for molecular technique-based species identification. (Lee et al., 2003) However, some of these genes have been found to lack specificity for *M.tuberculosis*. In addition, IS6110 PCR has been reported to produce false-negative (Yuen et al., 1993) and false- positive (Kent et al., 1995) results, and the *mtp40* gene is not present in all *M.tuberculosis* strains, (Weil et al., 1996).

Sequencing, DNA hybridization, PCR-restriction fragment length polymorphism analysis (RFLP) and microarray technology have also been employed to differentiate Mycobacterium species. (Lee et al., 2003).

Among the target genes, *rpoB* gene is of a particular interest as it comprises a highly conserved region throughout the eubacteria (Kim et al., 1999). It has also been suggested that the use of a novel duplex PCR method and sequence analysis of *rpoB* gene were a possible means of differentiation of *M.tuberculosis* complex and NTM species, as well as its use in the differentiation between species within the genus Mycobacterium. (Lee et al., 2003).

Accordingly, the present study **aimed** at evaluating the efficiency and usefulness of a Duplex PCR assay (DPCR) targeting *rpoB* gene to differentiate *M.tuberculosis* and NTM strains isolated from suspected cases of pulmonary tuberculosis in comparison with conventional culture based methods. Sequencing and restriction fragment length polymorphism (RFLP) were used to assist in the further identification of NTM strains.

The protocol of this study was approved by the faculty of Medicine, Alexandria University Ethics Committee prior to its start.

2. Materials and Methods

Mycobacterial isolates:

Forty one strains of mycobacteria constituted the material of this study. The strains were isolated from sputum samples of suspected pulmonary tuberculosis patients. The isolation was carried out in the "Tuberculosis Unit" of the Routine Microbiology Laboratory, Medical Microbiology and Immunology Department, Faculty of Medicine, Alexandria University as follows: sputum samples were decontaminated using Petroff's method (Petroff, 1915), the processed specimens were inoculated on Lowenstein Jensen (LJ) medium. The inoculated tubes were incubated at 37^0C and then inspected weekly for 8 weeks. All acid fast bacilli (AFB) isolates were assessed to distinguish between *M.tuberculosis* and NTM, according to (Kubica et al., 1963; Sommers et al., 1968): growth rate (strains that required more than 7 days to grow were considered slow growers), acid-alcohol fast staining, colonial morphology on (LJ) medium, preference of growth at 35^0C or 37^0C, photo reactivity, accumulation of niacin, growth on media containing 500 µg/ml Para-nitro benzoic acid.

DPCR using rpoB gene:

DNA extraction: A mycobacterium colony was suspended in 500µl of sterile distilled water, the mixture was heated at 100^0C for 30 minutes to kill the cells and induce lysis. This was followed by centrifugation and the supernatant containing DNA was collected and stored at -20^0C until use (Afghani and Stutman, 1996).

DNA amplifications were performed in a 25µl reaction mixture containing: 5µl of DNA extract, 12.5µl universal master mix (fermentas), 1µl (10 pmol) Tbc1 (5'-CGT ACG GTC GGC GAG CTG ATC CAA-3'), 1µl (10 pmol) TbcR5 (5'-C CAC CAG TCG GCG CTT GTG GGT CAA-3'), 2µl (20 pmol) M5(5'G GAG CGG ATG ACC ACC CAG GAC GTC-3'), 2µl (20 pmol) RM3 (5'CAG CGG GTT GTT CTG GTC CAT GAA C-3') and1.5µl nuclease free water. The thermal cycler was programmed as follows: Initial denaturation at 95^0C for 5 minutes, 30 cycles of denaturation at 95^0C for 30 seconds, annealing at 55^0C for 60 seconds and extension at 72^0C for 60 seconds and additional extension step at 72^0C for 5 minutes. The 235 bp amplicon of *M. tuberculosis*-specific primer set (Tbc1-TbcR5) and the 136 bp amplicon of NTM-specific primer set (M5-RM3) were run on 1.5% agarose gel and visualized using ethidium bromide staining (Kim et al., 2004). The specificity of each primer was assessed by a separate PCR. When a PCR using each primer set (Tbc1- TbcR5) or (M5- RM3) was applied to all test strains, at the same PCR conditions, an amplicon of either 235 or 136 bp of DNA was observed from *M.tuberculosis* or NTM strains respectively. When this method was applied to a negative control strain of *E.coli*, nothing was amplified (Kim et al., 2004).

For further identification of the 4 NTM strains, their 136 bp PCR product were subjected independently to the following:

DNA Sequence analysis:

The PCR products were purified to remove unincorporated nucleotides and excess primers using Wizard SV Gel and PCR clean-up system (Promega, USA). The forward and reverse strands of the purified PCR products were sequenced using the automated DNA sequencer (3130 X/ Genetic Analyzer, Applied Biosystems, Hitachi, Japan). The sequences obtained were aligned with the closest relatives retrieved from BLAST nucleotide database (http:www.ncbi.nlm.nih.gov/BLAST/) to identify the bacterial isolates tentatively.

RFLP:

On the basis of the *rpoB* sequence of mycobacteria (GenBank accession no. AF057449 to AF057493), two restriction enzymes, *MspI* and *HaeIII* were selected by using MapDraw (version 3.14; DNASTAR). The 2 restriction enzymes *MspI* and *HaeIII* (Takara, Shiga, Japan) were independently applied to the 136 bp PCR products, obtained from atypical (NTM) strains, as follows: 10µl PCR product, 2 U of each enzyme and restriction buffers were transferred to fresh microcentrifuge tube and nuclease free water was added to a final volume of 20 µl per reaction mixture. The reaction mixture was incubated at 37°C for 2 hours followed by chilling on ice. The obtained RFLP pattern was checked on 3% agarose gel using ethidium bromide staining and was analyzed by simple visual comparison with molecular weight marker. The pattern was interpreted using the published algorithm (Kim et al., 2004).

3. Results

Cultural and Biochemical Results: Thirty seven out of 41 strains (90.24%) were identified as *M. tuberculosis* because they were slow growers, non-pigmented, failed to grow at room temperature and on media containing PNBA, and showed positive niacin accumulation test. The remaining 4 (9.76%) isolates were identified as NTM because of their ability to grow on media containing PNBA and showing negative Niacin accumulation test. Regarding their rate of growth, pigmentation and growth at room temperature: 2 were slow growers and pigmented (one photochromogen and the other scotochromogen (that grew in a mixed culture with a typical *M. tuberculosis* isolate) but failed to grow at room temperature. The remaining 2 were rapid growers, non- pigmented and grew at room temperature. Several trials failed to separate the 2 mixed isolates in separate cultures.

DPCR Results: The 36 (90%) single mycobacteria isolates proved by phenotypic methods to be M. tuberculosis, produced 235 bp amplicon and were considered M. tuberculosis strains. The 3 (7.5%) single mycobacteria isolates proved by phenotypic methods to be NTM, produced 136 bp amplicon and were considered NTM. The two slow growers that coexisted in a single mixed culture and previously diagnosed phenotypically as a M. tuberculosis strain and a scotochromogen NTM strain, produced 2 amplicons (235 and 136 bp) in a single reaction. (Figure 1a,1b).

Table (1) shows the results of evaluation of DPCR assay in comparison to conventional phenotypic methods for identification of the 41 mycobacteria isolates. The sensitivity, specificity, positive predictive value and negative predictive value of DPCR were 100%, 100%, 100% and 100% respectively. The accuracy was 100%. The kappa value was 1.0 which reflects excellent agreement of DPCR results with those of the conventional methods.

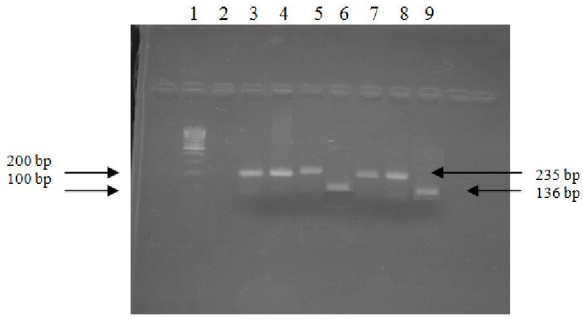

Figure 1a: Agarose gel (1.5%) electrophoresis showing PCR product of DPCR using rpoB gene in lanes 3-9: Lane 1 shows a 100bp molecular weight marker (M). Lanes 3,4,5,7 and 8 show the 235 bp amplicon of *M.tuberculosis* strains, while the 136 bp amplicon of non-tuberculous mycobacteria (NTM) is shown in lanes 6 and 9.

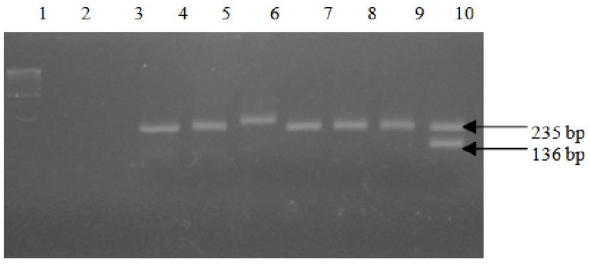

Figure 1b: Agarose gel (1.5%) electrophoresis showing PCR product of DPCR using rpoB gene in lanes 4-10: Lane 1 shows a 100bp molecular weight marker (M). Lanes 4 to 9 show the 235 bp amplicon of *M.tuberculosis* strains, while lane 10 shows both 235 and 136 bp amplicons of *M.tuberculosis* and non-tuberculous mycobacteria (NTM) respectively.

Table 1: Comparison between the *rpoB* gene DPCR results and the conventional cultural and biochemical results in mycobacteria identification:

		Conventional methods		Sensitivity	Specificity	PPV	NPV	Accuracy
		NTM	***M.tuberculosis***					
DPCR	**NTM**	TN = 4	FP = 0	100.0	100.0	100.0	100.0	100.0
	M.tuberculosis	FN = 0	TP = 37					

TN: true negative, FP: false positive, FN: false negative, TP: true positive, PPV: positive predictive value, NPV: negative predictive value.

Sequencing results

Depending on the BLAST results of forward strands of the 4 NTM isolates (according to the highest query coverage, lowest E-value and highest maximum identity), NTM strain (1) was expected to be *Mycobacterium gordonae,* NTM strain (2) was expected to be *Mycobacterium Kansasii,* NTM strain (3) *Mycobacterium abscessus and* NTM strain (4) *Mycobacterium chelonae.*

RFLP analysis results:

NTM strain (1) was digested by both enzymes (Hae III and MspI) into bands shorter than 100 bp in length. NTM strain (2) was digested by enzyme (Msp I) into bands shorter than 100 bp in length, while NTM strains (3 and 4) were not digested by these enzymes. (Figure: 2a,2b).

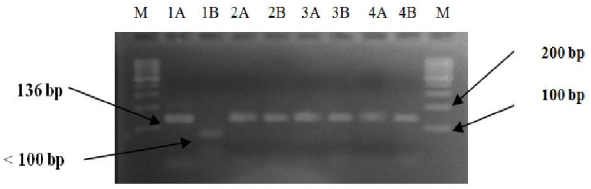

Figure 2a: Agarose gel (3%) electrophoresis showing the RFLP using the restriction enzyme *Hae III* for amplified PCR product from the four NTM strains. Samples were labeled NTM strain 1, 2, 3 and 4. A refers to the PCR product before cutting and B refers to the PCR product after cutting with the enzyme. M refers to 100 bp DNA marker

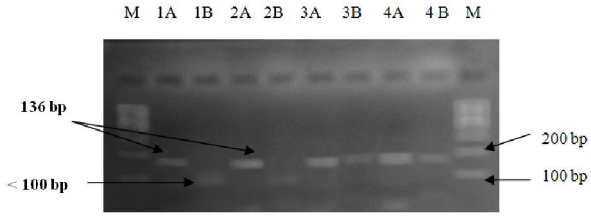

Figure 2b: Agarose gel (3%) electrophoresis showing the RFLP using the restriction enzyme *MspI* for amplified PCR product from four NTM strains. Samples were labeled NTM strain 1, 2, 3 and 4. A refers to the PCR product before cutting and B refers to the PCR product after cutting with the enzyme. M refers to 100 bp DNA marker.

4. Discussion

Mycobacteria identification to species level not only is of academic interest, but also is important because it provides a great deal of useful information on the epidemiology and pathogenesis of the organism, suggesting potential intervention strategies, including successful treatment of patients on a clinical basis (Springer et al., 1996).

There is an increasing demand for rapid, sensitive, and specific diagnostic methods for the detection and identification of *M. tuberculosis* and NTM in a clinical setting (Kim et al., 2004).

In the present study, 41 mycobacteria isolates from suspected cases of pulmonary tuberculosis were subjected to different phenotypic and genotypic testing methods aiming to differentiate *M. tuberculosis* from NTM isolates.

Results of phenotypic identification showed that out of 37 slow growers, 36 were identified as *M. tuberculosis* and one as NTM. The 2 rapid growers were identified as NTM. One culture showed slow growth of 2 mycobacteria isolates and were identified as a mixed culture of one *M. tuberculosis* and one NTM. The rate of detection of NTM in this study was about 9.76%.

Globally, the rate of detection of NTM in clinical specimens was generally higher, where NTM accounted for 13-25% of the total isolated mycobacteria in the period from 1999-2004 in Prague which could be attributed to higher incidence of HIV (Polanecky et al., 2006). Similar rates were detected in New Zealand in 2004 (Freeman et al., 2007). In England, Wales and Northern-island, the rate of NTM reports rose from 0.9 per 100,000 population in 1995 to 2.9 per 100,000 in 2006 (Moore et al., 2010). In USA, population-based surveys conducted during 1981–1983 estimated the prevalence of pulmonary NTM disease at 1–2 cases/100,000 persons in the United States (Billinger et al, 2009). A retrospective analysis from Ontario, Canada found an average annual increase of 8.4% for the isolation prevalence of NTM at the Ministry of Health Mycobacterial Laboratory between 1997 and 2003 (Marras et al., 2010). In Taiwan, from January 2000 through December 2008, NTM represented 3.2% of isolated cases (Lai et al., 2010).

In Egypt, NTM accounted for 10% of the total isolated mycobacteria as detected by El-Hefnawy (1989), higher rates were detected by Shoukry et al.

(1995), and Rizk et al. (1998) (18.6% and 14% respectively). El- Haddad (1999) detected a rate of 7.14%, while Okasha (2003) detected a rate of 11%.

Due to their phenotypic similarity and difficulty in characterization by conventional methods of identification (Kim et al., 1999), methods capable of simultaneous identification of *M. tuberculosis* and/or NTM would be useful. A novel Duplex PCR technique targeting single gene such as *rpoB* gene and *hsp65* was developed to differentiate between *M. tuberculosis* and NTM (Kim et al., 2004). The present study evaluated the efficiency and usefulness of Duplex PCR assay targeting *rpoB* gene to differentiate *M. tuberculosis* complex and NTM strains isolated from cases of pulmonary tuberculosis, in comparison to conventional culture and biochemical (phenotypic) methods.

The DPCR results showed that the 36 (90%) single mycobacteria isolates proved by phenotypic methods to be *M. tuberculosis* complex strains produced 235 bp amplicon and were considered *M. tuberculosis* complex. In addition 3 (7.5%) single mycobacterial isolates proved by phenotypic methods to be NTM produced 136 bp amplicon and were considered NTM. When DPCR was performed on a mixed culture, 2 amplicons were produced; 235 and 136 bp. This mixed culture was identified phenotypically as mixed growth of two slow growers; *M. tuberculosis* complex and NTM. Comparison of DPCR and phenotypic results showed excellent (100%) agreement.

These results agreed with Kim et al. (2004) who used the same duplex PCR method based on *rpoB* gene using the same two sets of primers on 44 reference mycobacterial strains and 379 mycobacterial isolates. They reported that this duplex PCR method clearly differentiated all *M. tuberculosis* and NTM strains as 235 bp DNAs were amplified from the *M. tuberculosis* complex strains and 136 bp DNAs were amplified from NTM strains. These results were completely concordant with those obtained by conventional methods.

Moreover, one of the advantages of DPCR analysis stated by Kim et al. (2004) was realized in the present study, where one culture containing mixed growth (suggesting the coexistence of *M. tuberculosis* and NTM), yielded 2 PCR products (136 & 235 bp) in the same reaction mixture.

Sequencing results obtained from the 136 bp amplicons produced from the 4 NTM strains were in complete harmony with the phenotypic results.

As regards the RFLP results of the present study, they were in complete concordance with results of Kim et al. algorithm (2004) as follows: strain (1) (that was previously expected by sequencing to be *M. gordonae*), was digested by both *Hae III* and *Msp I* enzymes and produced bands < 100 bp in length, fell in the group that contained *M. gordonae, M. intracellulare* and *M. intermedium* in the algorithm. Similarly, strain (2) (previously expected by sequencing to be *M. kansasii*), was digested by *Msp I* enzyme and produced bands < 100 bp fell in the group that contained *M. kansasii, M. marinum, M. ulcerans, M. trivial* and *M. shimoidei* in the algorithm. Finally, strain (3) and (4) (that were previously expected by sequencing to be *M. abscessus* and *M. chelonae* respectively) were not digested by either enzyme and fell in the group that contained *M. abcessus, M. aurum, M. chelonae, M. flavescence, M. fallax, M. fortuitum, M. pheli, M. peregninum, M. vacce and M. smegmatis* in algorithm.

In Conclusion, DPCR assay based on rpoB gene with two different sets of primers provides a rapid and reliable mean for the differential identification of M. tuberculosis and NTM isolates in culture with a single step assay. The two sets of primers could amplify two different sized DNA from a single target gene. Even coexistence of M. tuberculosis and NTM could be detected by the presence of two different PCR products in a single reaction mixture. Further studies are recommended to determine the applicability of this DPCR assay on clinical specimens, and to evaluate other molecular techniques targeting genes such as *hsp65* in comparison with *rpoB* gene, to overcome the disadvantage of this DPCR that has been reported previously [195] which is the short length of the NTM amplicon (136 bp) constituting a problem in developing restriction analysis or direct sequencing protocol that fully identifies NTM strains, allowing only for possible grouping and not complete species identification.

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