ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research 2 (6) 2013, 30-34

RESEARCH ARTICLE

IDENTIFICATION OF CLINICALLY RELEVANT MYCOBACTERIUM OTHER THAN TUBERCULOSIS (MOTT) SPECIES BY REAL-TIME PCR COUPLED WITH A HIGH-RESOLUTION MELTING SYSTEM

Prem Raj Singh[1], Devender Singh Gill[1], Preeti Caesar[1], Veena Sharma[*2]

[1]Auroprobe Laboratories NH-58 Muradnagar, Ghaziabad (U.P), India.

[2*] Department of Bioscience and Biotechnology, Banasthali University, Banasthali, Rajasthan, India.

Received 10 October 2013; Revised 22 October 2013; Accepted 25 October 2013

ABSTRACT

A real-time PCR coupled with a high-resolution melting curve assay for identifying clinically relevant MOTT species was developed and evaluated using 6 reference strains and 186 Culture isolates. Concordant results were obtained for all 186 isolates of nontuberculous mycobacteria. Our results showed that this real-time PCR -HRM assay is an effective tool for the mycobacterial identification from cultures isolates.

Key words: MOTT (Mycobacterium other than tuberculosis**),** HRM (high-resolution melting), Real time PCR.

INTRODUCTION:

In spite of long-lasting strong efforts to overcome tuberculosis (TB), this disease continues to contribute to an intensifying global health crisis (1). It has always been endemic and it is probably due **to** the nontuberculous mycobacteria (NTM). These other mycobacteria were previously known by different names as 'atypical', 'anonymous', 'Mycobacteria Other Than Tuberculosis' (MOTT) or Potentially Pathogenic Environmental Mycobacteria (PPEM). But today the terminology of Non-Tuberculosis Mycobacterium (NTM) given by the International Working Group on Mycobacterial Taxonomy is universally accepted. Genus Mycobacterium has 130 well characterized species (2). Most of Mycobacteria other than tuberculosis (MOTT) are present in the environment as saprophytes except for *Mycobacterium tuberculosis* (which causes the disease tuberculosis (TB) and *Mycobacterium leprae* (which causes leprosy). The rate of isolation of mycobacteria other than tuberculosis (MOTT) has increased over the past several years; in some areas the isolation rate for *Mycobacterium avium, Mycobacterium intracellulare* has exceeded that for *M. tuberculosis*. Simultaneously, the spectrum of clinical manifestations associated with the various species has widened. (3)

As the culture with strict criteria is still not routinely done in most parts of India and there is a tendency to overlook such isolates as contaminants, it would be hard to comment on the exact magnitude of the problem. NTM have been observed to be an important cause of morbidity and mortality in Western countries and India. The rapid detection and identification of clinically important *Mycobacterium* species is essential for patient management and infection control. Speciation is particularly important when choosing antibiotic regimens for immunocompromised patients, in whom the presence of any acid-fast bacilli may be considered significant.Traditional methods for the detection of mycobacteria, such as the acid-fast stain and culture, have either a low sensitivity or specificity, or take a long time for final result. Therefore a rapid diagnostic method is necessary which combines high sensitivity and high specificity.

Detection and identification of clinically relevant MOTT species by PCR-based methods have described in some previous studies. (4,5,6). Multiplex PCR, which simultaneously uses two or three different genes, has been commonly used, as the technique can specially detect and identify different species of the genus Mycobacterium (7,8,9) and differentiate members of the M. tuberculosis complex (9) in the routine diagnostic laboratory by using Mycobacterium genus- and species-specific genes. However, PCR-based methods either multiplex PCR or PCR coupled with other techniques (e.g. restriction enzyme digestion, sequencing, microarray analysis and mass spectrometry) for rapid identification of MOTT are still long and require many bulky procedures to differentiate the MOTT species (10,11,12,13). Several PCR-based technologies for rapid identification of MOTT are commercially available but these are too costly for most clinical laboratories and poor country like Bangladesh, India etc. (14, 15, and 16). High Resolution Melting (HRM) is a novel, close-tube, homogeneous,

[*]**Corresponding author:** Dr. Veena Sharma **| Email:** veena.sharma61@gmail.com

post-PCR method, facilitating genomic researchers to investigate genetic variations (SNPs, mutations, methylations) in PCR amplicons with various saturation dyes such as LC Green, SYTO9 and Eva Green (17). According to the objective this study was to establish a combined real-time PCR-HRM assay for common clinically relevant MOTT species identification. The performance of the combined real-time PCR-HRMA assay was compared with that of the 16S rRNA gene sequencing method.

MATERIALS AND METHODS:

Reference strains and clinical isolates:

186 MOTT isolates were obtained from culture positive clinical specimens by NAP test from November, 2010 to November, 2012 at the Auroprobe laboratories. The reference strains used in this study were *M. kansassi* clinical strain, *M. avium* clinical strain, *M. fortuitum* clinical strain, *M. chelonae* clinical strain, *M. abscessus* clinical strain, *M. intracellure* clinical strain. Ethical approval was not needed for the current study as all the samples from the subjects were received for clinical diagnosis from Different collection points and we had not disclosed any identification of the subjects.

Sample processing and MOTT identification:

A total of 3066 clinical specimens were processed between November- 2010 to November -2012. All clinical specimens were digested and decontaminated by theN-acetyl-L-cysteine–NaOH method as described by Sun et al. (18). A 4% concentration (starting concentration) of NaOH was used. The remaining sediment was suspended in 1.5 ml of sterile phosphate-buffered saline (pH 6.8). Before inoculation, BACTEC 12B vials were supplemented as described by the manufacturer. All specimens were processed and decontaminated within 24 h after collection, as described previously (19). Total nucleic acids were extracted from culture positive BACTEC 12B vials for evaluation of real-time PCR-HRMA and for full-length 16S rRNA gene sequencing, respectively.

Primer pair selection:

From among the 4 primer pairs initially evaluated, 1 primer pair candidates were selected based on their *in silico* sensitivities and specificities for mycobacterial DNA amplification (data not shown). Primers targeted to the 16– 23S internal transcribed spacer (ITS) region, RNA polymerase gene (rpoB), 16S rRNA gene, or hsp65 gene have been described in relation to detecting mycobacteria by PCR in previous publications. We used Primer Express Software v2.0 to confirm the conservativeness and specificity of these primers for mycobacteria.

Real-time PCR:

The oligonucleotide primers for PCR targeted for amplification a portion of the mycobacterial 16S rRNA gene. The sequence of the forward primer was 5-ATGCAAGTCGAACGGA-3, whereas the sequence of the reverse primer was 5- GTCGTCGCCTTGGTAG -3. Real-time PCR was performed with a melt doc kit (Applied Biosystems) on an ABI 7500 fast Real-Time PCR system. Each PCR analysis contained one primer pair and cycling conditions were as follows: denaturation at 95^{0}C for 10 min, followed by 50 cycles of denaturation at 95^{0}C for 15 s and annealing/extension at 60 ^{0}C for 30 s, with a final cycle at 95 ^{0}C for 30 s and 28 ^{0}C for 30 s.

High-Resolution Amplicon Melting:

Each real-time PCR amplicon was subjected to HRMA on ABI 7500 fast Real-Time PCR system and analyzed using HRM software version 2.0.

DNA Sequencing:

Real time PCR products were sequenced by Ocimum Biosolutions, Ltd. (DATA NOT SHOWN)

RESULTS:

During the 12 month study period, we identified 641 AFB positive BACTEC culture from 3066 clinical specimens. We differentiate MOTT and MTB from AFB culture positive samples by using the NAP test. A total of 186 samples were found MOTT positive by NAP tested and use for Real time PCR HRM assay. All samples were send to Ocimum Biosolutions, Ltd to analyzed full-length sequencing of the 16S rRNA gene (data not shown). The DNA extracted from MOTT-positive culture vial was subjected separately to real-time PCR of the 16S rRNA gene. The melting curves of the isolates were demonstrated to be reported from run to run. Of the 186 isolates analyzed and difference plots of the real-time PCR-HRM of 16S rRNA gene were classified into six groups. In these four major clusters, 57 isolates were *Mycobacterium avium*, 23 isolates were *M. chelonae*, 12 isolates were *M. fortuitum*, 10 isolates were *Mycobacterium abscessus,* 19 isolates were *Mycobacterium intracellure* and 29 isolates were *Mycobacterium kansasai*.

© 2012 JBPR. All Rights Reserved.

Table: 1 Sensitivity and specificity of HRM Real time PCR assay

Species Group and species	No. of isolates				Sensitivity (%)	Specificity (%)	NPV (%)	PPV (%)
	TP	TN	FP	FN				
M. avium	53	89	1	3	94.64%	98.88%	96.73%	98.14%
M. chelonae	23	126	1	2	92.00%	99.21%	98.43%	95.83%
M. fortuitum	12	120	2	1	92.30%	98.36%	99.17%	85.71%
M. kansasai	29	134	1	2	93.54%	99.25%	98.52%	96.66%
M. abscessus	10	139	0	0	100%	100%		
M. Intracellure	19	135	1	1	95.00%	99.26%	99.26%	95.00%

Out of 186 samples only 161 clinical MOTT isolates were identified in terms of species by full 16S rRNA gene sequencing (table -2) as a standard method while 25 samples were failed in sequencing. Overall, 146 of the 161 MOTT (90.68 %) were successfully identified into 6 species by Real time PCR-HRM assay, comprising *M. avium* (53), *M. chelonae* (23), *M. fortuitum* (12) and *M. kansasai* (29), *M. abscessus* (10), *M. intracellure* (19). Of these 134 prevalent mycobacterial isolates, 6 mycobacteria could be identified correctly by real-time PCR-HRM assay. The individual sensitivities for M. avium, M. chelonae, *M. fortuitum* and *M. kansasai*, *M. abscessus*, *M. intracellure* were 94.64%, 92.00%, 92.30% , 93.54 %, 100%, and 95.00% respectively (Table 1). The individual specificities for M. avium, M. chelonae, *M. fortuitum* and *M. kansasai*, *M. abscessus*, *M. intracellure were* 98.88%, 99.21%, 98.36% 99.25%, 100%, and 99.26% respectively (Table 1).

Table: 2 Comparison of identification of 161 MOTT isolates by real-time PCR-HRMA and 16S rRNA gene full-length sequencing

MOTT Species identified by 16S rRNA gene sequencing	MOTT isolates identified by real-time PCR-HRMA
M. avium 57	53
M. chelonae 26	23
M. fortuitum 15	12
M. kansasai 32	29
M. abscessus 10	10
Ml. intracellure 21	19

DISCUSSION:

Due to the unlike pathogenic potentials and susceptibilities of MOTT from M. tuberculosis, drugs of choice for the treatment of MOTT infections differ (20). Early identification of the species of mycobacterium causing illness in a patient would have significant clinical impact. (21) Species identification mycobacterium is important for surveillance and suitable choice of antibiotics but often is not performed because of technical difficulty. Real-time PCR has been used to differentiate members of the M. tuberculosis complex from Mycobacterium other than tuberculosis MOTT (22, 23, and 24). This assay identified and distinguished different clinically relevant MOTT species. This represents an advance in our ability to rapidly identify MOTT isolates without the added costs of additional hybridization probes. This test would also give information suggestive of the type of NTM present, which possibly could help in subsequent test selection.

© 2012 JBPR. All Rights Reserved.

CONCLUSION:

Therefore the real-time PCR-HRM assay is a rapid and sensitive method for identifying clinically relevant MOTT species.

ACKNOWLEDGMENT:

The authors are thankful to Laboratory technical staffs of Auroprobe Laboratories NH-58 Muradnagar, Ghaziabad, India for helping in this study.

REFERENCE:

1. World Health Organization. (1997). WHO report on the tuberculosis epidemic. WHO/TB/97.225. World Health Organization, Geneva, Switzerland.
2. Katoch VM (2004). Infections due to non-tuberculous mycobacteria (NTM). Indian J Med Res. 120:290-304.
3. American Thoracic Society. (1997). Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am. J. Respir. Crit. Care Med. 156:S1–S25.
4. Li, H., Turhan, V., Chokhani, L., Stratton, C. W., Dunbar, S. A. & Tang, Y.-W. (2009). Identification and differentiation of clinically relevant Mycobacterium species directly from acid-fast bacillus-positive culture broth. J Clin Microbiol 47, 3814–3820.
5. Kim, K., Lee, H., Lee, M.-K., Lee, S.-A., Shim, T.-S., Lim, S. Y., Koh, W.-J., Yim, J.-J., Munkhtsetseg, B. & other authors (2010).Development and application of multiprobe real-time PCR method targeting the hsp65 gene for differentiation of Mycobacterium species from isolates and sputum specimens. J Clin Microbiol 48, 3073– 3080.
6. Shrestha, N. K., Tuohy, M. J., Hall, G. S., Reischl, U., Gordon, S. M. & Procop, G. W. (2003). Detection and differentiation of Mycobacterium tuberculosis and nontuberculous mycobacterial isolates by real-time PCR. J Clin Microbiol 41, 5121–5126.
7. Del Portillo, P., M. C. Thomas, E. Martínez, C. Maran˜on, B. Valladares, M. E. Patarroyo, and M. C. Lo´pez (1996). Multiprimer PCR system for differential identification of mycobacteria in clinical samples. J. Clin. Microbiol. 34:324–328.
8. Klemen, H., A. Bogiatzis, M. Ghalibafian, and H. H. Popper (1998). Multiplex polymerase chain reaction for rapid detection of atypical mycobacteria and Mycobacterium tuberculosis complex. Diagn. Mol. Pathol. 7:310–316.
9. Herrera, E. A., O. Perez, and M. Segovia (1996). Differentiation betweenMycobacterium tuberculosis and Mycobacterium bovis by a multiplex-polymerase chain reaction. J. Appl. Bacteriol. 80:596–604.
10. Wu, T.-L., Chia, J.-H., Kuo, A.-J., Su, L.-H., Wu, T.-S. & Lai, H.-C. (2008). Rapid identification of mycobacteria from smear-positive.
11. Hall, L., Doerr, K. A., Wohlfiel, S. L. & Roberts, G. D. (2003). Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol 41, 1447–1453.
12. Lim, S. Y., Kim, B.-J., Lee, M.-K. & Kim, K. (2008). Development of a real-time PCR-based method for rapid differential identification of Mycobacterium species. Lett Appl Microbiol 46, 101–106.
13. Wu, T.-L., Chia, J.-H., Kuo, A.-J., Su, L.-H., Wu, T.-S. & Lai, H.-C. (2008). Rapid identification of mycobacteria from smear-positive sputum samples by nested PCR-restriction fragment length polymorphism analysis. J Clin Microbiol 46, 3591–3594.
14. Padilla, E., Gonza´ lez, V., Manterola, J. M., Pe´ rez, A., Quesada, M. D., Gordillo, S., Vilaplana, C., Pallare´ s, M. A., Molinos, S. & other authors (2004). Comparative evaluation of the new version of the INNO-LiPA Mycobacteria and genotype Mycobacterium assays for identification of Mycobacterium species from MB/BacT liquid cultures artificially inoculated with mycobacterial strains. J Clin Microbiol 42, 3083–3088.
15. Lebrun, L., Weill, F. X., Lafendi, L., Houriez, F., Casanova, F., Gutierrez, M. C., Ingrand, D., Lagrange, P., Vincent, V. & Herrmann, J. L. (2005). Use of the INNO-LiPA-MYCOBACTERIA assay (version 2) for identification of Mycobacterium avium-Mycobacterium intracellulare- Mycobacterium scrofulaceum complex isolates. J Clin Microbiol 43, 2567–2574.
16. Hall, L., Doerr, K. A., Wohlfiel, S. L. & Roberts, G. D. (2003). Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical mycobacteriology laboratory. J Clin Microbiol 41, 1447–1453.
17. Cheng, J.-C., Huang, C.-L., Lin, C.-C., Chen, C.-C., Chang, Y.-C., Chang, S.-S. & Tseng, C.-P. (2006). Rapid detection and identification of clinically important bacteria by high-resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clin Chem 52, 1997–2004.
18. Sun, J.-R., Lee, S.-Y., Perng, C.-L. & Lu, J.-J. (2009). Detecting Mycobacterium tuberculosis in Bactec MGIT 960 cultures by inhouse IS6110-based PCR assay in routine clinical practice. J Formos Med Assoc 108, 119–125.

© 2012 JBPR. All Rights Reserved.

19. Huang TS, Chen CH, Lee SS, Huang WK, LiuY C (2001). Comparison of BACTEC MGIT 960 and BACTEC 460 TB System for Detection of MyCobacteria in Clinical Specimens. Annals of Clinical and Laboratory Science. 31:279-83.
20. Wolinsky, E. (1992). Mycobacterial diseases other than tuberculosis. Clin. Infect. Dis. 15:1–10
21. Thierry, D., A. Brisson-Noel, V. Vincent-Levy-Frebault, S. Nguyen, J. L. Guesdon, and B. Gicquel. (1990). Characterization of a Mycobacterium tuberculosis insertion sequence, IS6110, and its application in diagnosis. J. Clin. Microbiol. 28:2668–2673.
22. Foongladda, S., Pholwat, S., Eampokalap, B., Kiratisin, P. & Sutthent, R. (2009). Multi-probe real-time PCR identification of common Mycobacterium species in blood culture broth. J Mol Diagn 11, 42–48.
23. Richardson, E. T., Samson, D. & Banaei, N. (2009). Rapid Identification of Mycobacterium tuberculosis and nontuberculous mycobacteria by multiplex, real-time PCR. J Clin Microbiol 47, 1497– 1502.
24. Shrestha, N. K., Tuohy, M. J., Hall, G. S., Reischl, U., Gordon, S. M. & Procop, G. W. (2003). Detection and differentiation of Mycobacterium tuberculosis and nontuberculous mycobacterial isolates by real-time PCR. J Clin Microbiol 41, 5121–5126.

© 2012 JBPR. All Rights Reserved.