Identification of Several Mycobacterium Species by Using Amplified 16S Ribosomal DNA

Niharika M. Amrute, Atul P. Denge, Dilip G. Gore, and Suchitra S. Godbole

***Abstract*—In order to diagnose MOTT species *M. smegmatis* and M. kansasii along with M. tuberculosis, species specific primers were designed using PRIMER-BLAST which amplified particular 16S rRNA genes having variable amplicons sizes. All primer sets were found to be specific for their target 16S regions of mycobacterium species. Easy and fast DNA isolation protocol developed that could be implemented directly for PCR reaction of the samples. By involving standardized PCR conditions with defined primers and genome combinations, we have successfully reported the 16S rRNA gene based PCR detection of several mycobacterium species which has highlighted the MOTT detection capabilities.**

***Index Terms*—MOTT, Mycobacterium species, PCR, 16S rRNA.**

I. Introduction

The pathogenicity of *Mycobacterium tuberculosis* is known worldwide and proves to be epidemic to HIV positive patients [1]-[3]. Severity also carries with mycobacterium other than *Mycobacterium tuberculosis* (MOTT) mainly with *Mycobacterium smegmatis* and *Mycobacterium Kansasii [4]*,[5]. *M. smegmatis* is known as environmental mycobacterium first identified in 1884, yet is a rare pathogen in humans. The few *M. smegmatis* infections reported and those occurred in association with a primary lesion in otherwise immunocompetent individuals. Studies evidenced *M. smegmatis* acts as a new opportunistic agent that may be responsible for disseminated disease in immunocompromised individuals [6]. In several cases, *M. kansasii* reported for an endobronchial, ulcerated lesion and in view various methods were suggested for infection diagnosis one of it was fiberoptic bronchoscopy [7].

Involving risk factors related to mycobacteria other than *Mycobacterium tuberculosis* are iatrogenic immunosuppression, preexisting lung disease e.g., chronic obstructive pulmonary disease, and a previous tuberculosis [8].

Atypical mycobacteria previously been recognized as saprophytic organisms for coming years, but with the advent microbiological culture techniques, those were recognized as potentially pathogenic to man [9]. Geographical distribution of MOTT sensitivity supported the theory that large bodies of water are the main source of infection with MOTT [10].

MOTT infection is a rare event but emerging problems of infliximab therapy. MOTT cases tend to progress rapidly in infliximab-treated patients and withdrawal of infliximab therapy can result in immune reconstitution [11]. The rate of isolation of MOTT has increased over the past several years,in some areas the isolation rate for *Mycobacterium avium-Mycobacterium intracellulare* has surpassed that for *M. tuberculosis*. Simultaneously, the spectrum of clinical manifestations with the various species has widened [12].

By looking at the severity of the MOTT, it is required to diagnose these MOTT species with respect to *Mycobacterium tuberculosis*. In concern, several methodologies were developed which encompasses traditional staining methods and several sophisticated techniques like PCR and ELISA. The differential diagnosis between tuberculosis (TB) and lymphadenitis caused by MOTT in children is often based on epidemiologic and clinical data. Where it was reported that such data was useful in the differential diagnosis between TB and NTM lymphadenitis when etiologic diagnosis was not available [13]. Technique like PCR-restriction fragment length polymorphism (PCR-RFLP) was used for the early detection and identification of *Mycobacterium tuberculosis* directly from clinical samples. PCR amplification of 16S rRNA gene sequence for the detection of several MOTT species against *M. tuberculosis* has been developed for early detection of the infection [2],[14]-[17].

In view, present study was planned for PCR based detection of *M. tuberculosis, M. smegmatis* and *M. kansasii* using several species specific primers and results were confirmed by homology study.

II. Materials and Methods

A. Culture Collection

The lyophilized pure cultures of *Mycobacterium tuberculosis* (mtb) (MTCC MTB 300), *Mycobacterium smegmatis* (msm) (MTCC MSM 06) and *Mycobacterium kansasii* (mka) (MTCC MKA 3058) were collected from Institute of Microbial Technology, Chandigarh, India.

B. Maintenance on L J medium

The lyophilized cultures were grown on Lowenstein-Jensen (LJ) media for three weeks at 37^{o}C for initial growth and then sub cultured after 25 days.

C. Isolation of Genomic DNA

The genomic DNA of mtb, mka and msm were isolated by using NaOH / Tris buffer protocol as mentioned below:-

A loopful culture from LJ media was put in microfuge tube (2.0 ml). Then 1.5 ml Tris EDTA buffer (10mM tris HCl, 1mM EDTA, pH 8.0) was added followed by vigorous pipetting which ensured proper distribution and separation of

Manuscript received August 19, 2011; revised September 10, 2011.

Authors are with Biotechnology and Microbiology department of G. H. Raisoni institute of Interdisciplinary Sciences Nagpur (email: acmedilip4u@yahoo.com)

the cultures in TE buffer. Tubes were further centrifuged at 10,000 rpm for 5 min. Obtained supernatants were decanted and pellet washed twice with sterile distilled water and drained. 25 µl of 0.5 N NaOH added further and incubated for 30 min. at room temperature. It was supplemented with 25 µl of Tris buffer and mixed properly. After mixing, 450 µl sterile distilled water was added and stored at -20°C for 1 hour or used immediately.

D. Primer Designing

Specificity and success of PCR generally depends on the primer sets used for the amplification of specific locus. In concern, the 16S rRNA gene sequences of mtb, msm and mka were used as a template to design the specific primer sets for each mycobacterium species. Respective primer set possessed specificity for mtb, mka and msm 16S ribosomal DNA. The Primer BLAST program was used to design the primer sets. In primer setting parameters, common forward primer was designed for all three mycobacterium studied while reverse primers brought about the specificity and differences in the amplicon sizes for 16S rRNA of each species. The web Address is: www.ncbi.nlm.nih.gov/tools/primer-blast/.

E. Polymerase Chain Reaction

The PCR reaction was carried out in the Applied Biosystem thermal cycler using 50µl reaction mixture comprised of PCR water (29µl), 10X reaction Buffer (05µl), Primers (06 µl), DNA template (05µl), dNTP mix (2.5µl) and Taq Polymerase (2.5µl).While the PCR condition was set as follows: - In which, initial hold up temp. set at 94°C for 3 min., denaturation at 94°C for 1.5 min., annealing at 65°C for 2 min., extension at 72°C for 3 min. and final hold up at 4°C and cycles were repeated for 40 times.

F. DNA Sequencing

The PCR amplicons were sequenced using 50ng of PCR product in a sequencing reaction that contained 8 µl of ready reaction mix (BDT v 3.0, Applied biosytems, Foster city, CA) and 5pmol of forward primer. The cycling condition was set at: 25 cycles of 96°C for 10 sec., 50°C for 5 sec. and 60°C 4 min. The sequencing was carried out in ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

III. Results

A. Culture Collection

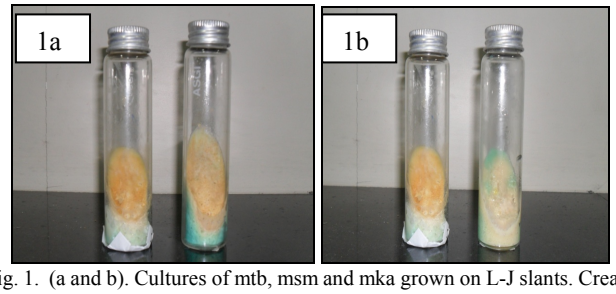

Fig. 1. (a and b). Cultures of mtb, msm and mka grown on L-J slants. Cream colored colonies (lawn) appeared after incubation.

The lyophilized cultures of mtb, msm and mka were successfully maintained on L-J media. The growth was apparent on each media with cream colored colonies appeared on the onset of 30th day (Fig.1).

B. Primer designing

By using the 16S ribosomal DNA sequences of mtb, mka and msm in PCR–BLAST using diagnostic based parameter settings, we have successfully retrieved forward and reverse primer sets. Those have demonstrated higher specificity and selectivity because of the non-availability of the binding site/s in the species other than interested. Specificity was confirmed by performing the nucleotide BLAST against the genome of *mycobacterium* excluding a genome to which primer belongs. The primer designing was intended to design two types of primer sets. One set was highly specific to all three *Mycobacterium* species and nominated as universal primers and named as CMYC20F and CMYC518R, while other sets were made specific according to mtb, mka and msm. This gave us the opportunity to screen the comparative sensitivity of each primer set with their respective genome. In study, diagnosis remained dependent on the reverse primers used, as forward primer is common to both the species. The designed primers were highlighted below in Table I.

C. Polymerase Chain Reaction

The PCR based diagnostics of msm and mka along with mtb could dramatically shift the diagnostic sensitivity and specificity to higher level. By analyzing results, it could be demonstrated that by using designed primers along with particular genome with mentioned PCR conditions, diagnosis of msm, mka and mtb is possible with higher specificity. The results were encouraging and explained its success in a greater detail (Fig. 2 and 3) (Table II and III). By using the specified primer sets as in tables II and III along with the particular genomes, results highlighted the specificity of the primers for their target sequences. For e.g. in lane 01 of Fig.2 and lane 2 of Fig.3, positive 16S amplicons were obtained when mtb genome was amplified with mtb specific Primers. While no amplifications were observed in lane 06 of Fig. 2 and lane 4 of Fig.3 where mtb genome was amplified with msm and mka specific primers respectively. These results have demonstrated the selectivity and specificity of the species specific primers for their templates and similar results were documented for msm as well as for mka (Fig. 2 & 3). Along with these three specific primer sets for mtb, msm and mka, another primer set was analyzed, these primers have detected all the three *mycobacterium* species, as amplification was observed with every genome tested this suggested that use of CMYC20F and CMYC518R, together could identify presence of these *mycobacterium* species (Fig 2 & 3).

D. Sequence data

The amplicons obtained for 16S specific PCR of mtb, msm and mka were sequenced and these were named as sequenced tagged sites (STS). Every STS reported a varying sequence length and reported below in detail:-

> STS 16S mtb 209 bp (mtb sp. primers CMYC20F + SMTB18R and mtb genome)

GAGATACTCGAGTGGCGAACGGGTGAGTAACACGT
GGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGG
AAACTGGGTCTAATACCGGATAGGACCACGGGATG
CATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTGG
GATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGT
GACGGCCTACCAAGGCGACGACGGGTAGCCGGCC

>STS 16S mka 114 bp (mka sp. primers CMYC20F + SKAN20R and mka genome)
TACTCGAGTGGCGAACGGGTGAGTAACACGTGGGC AATCTGCCCTGCACACCGGGATAAGCCTGGGAAAC TGGGTCTAATACCGGATAGGACCACTTGGCGCATG CCTTGTGGT

> STS 16S 149 bp (Common primers CMYC20F + CMYC518R and msm genome)
GGGGTACTCGAGTGGCGAACGGGTGAGTAACACGT GGGTGATCTGCCCTGCACTTTGGGATAAGCCTGGG AAACTGGGTCTAATACCGAATACACCCTGCTGGTC GCATGGCCTGGTAGGGGAAAGCTTTTGCGGTGTGG GATGGGCCC

> STS 16S 106 bp (msm sp. primers CMYC20F + SMSM19R and msm genome)
GGGGTACTCGAGTGGCGAACGGGTGAGTAACACGT GGGTGATCTGCCCTGCACTTTGGGATAAGCCTGGG AAACTGGGTCTAATACCGAATACACCCTGCTGGTC GCATG

> STS 16S mtb 149 bp (Common primers CMYC20F + CMYC518R and mtb genome)
GAGATACTCGAGTGGCGAACGGGTGAGTAACACGT GGGTGATCTGCCCTGCACTTCGGGATAAGCCTGGG AAACTGGGTCTAATACCGGATAGGACCACGGGATG CATGTCTTGTGGTGGAAAGCGCTTTAGCGGTGTGG GATGAGCCC

> STS 16S mtb 143 bp (Common primers CMYC20F + CMYC518R and mka genome)
TACTCGAGTGGCGAACGGGTGAGTAACACGTGGGC AATCTGCCCTGCACACCGGGATAAGCCTGGGAAAC TGGGTCTAATACCGGATAGGACCACTTGGCGCATG CCTTGTGGTGGAAAGCTTTTGCGGTGTGGGATGGG CCC

TABLE I. PRIMER SEQUENCE INFORMATION FOR MTB, MKA AND MSM 16S RRNA DNA

Primer names	Primer seq. 5'-3'	Length	Melting temp. °C	Mol. wt.
CMYC20F	GAGATACTCGAGTGGCGAAC	20	54	6191.05
SMTB18R	GGCCGGCTACCCGTCGTC	18	59	5452.51
SMSM19R	CATGCGACCAGCAGGGTGT	19	55	5853.8
SKAN20R	ACCACAAGGCATGCGCCAAG	20	56	6105.06
CMYC518R	GGGCCCATCCCACACCGC	18	59	5390.55

CMYC20F = common *mycobacterium* species 20 nucleotides forward primer
SMTB18R = specific mtb 18 nucleotides reverse primer
SMSM19R = specific msm 19 nucleotide reverse primer
SKAN20R = specific mka 20 nucleotides reverse primer
CMYC518R = common *mycobacterium* species 18 nucleotides reverse primer

TABLE II. PRIMER USED IN 16S RRNA GENE AMPLIFICATION OF MTB AND MSM

Lane	Primer type and genome used	Amplicon size (bp)
M	100 bp DNA ladder	___
1	mtb sp. Primers with mtb genome (CMYC20F + SMTB18R)	209
2	Common primers with mtb genome (CMYC20F + CMYC518R)	149
3	Mtb sp. Primer with msm genome	No
4	msm sp. primers with msm genome	106
5	Common primers with msm genome	149
6	msm sp. primers with mtb genome	No

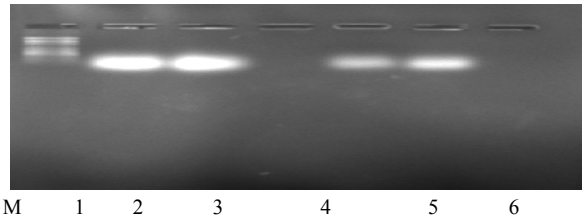

Fig. 2. 16S Ribosomal DNA PCR amplicons of mtb and msm resolved on 1 % agarose gel as per table II.

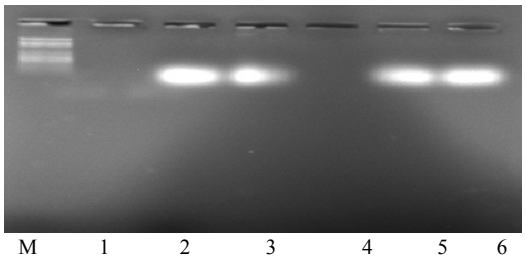

Fig. 3. 16S Ribosomal DNA PCR amplicons of mtb and mka resolved on 1 % agarose gel as per table III.

TABLE III. PRIMER USED IN 16S RRNA GENE AMPLIFICATION OF MTB AND MKA

Lane	Primer type and genome used	Amplicon size (bp)
M	100 bp DNA ladder	___
1	Mtb sp. Primers with mka genome (CMYC20F + SMTB18R)	No
2	mtb sp. primers with mtb genome (CMYC20F + SMTB18R)	209
3	Common primers with mtb genome (CMYC20F + CMYC518R)	149
4	mka sp. primers with mtb genome (CMYC20F + SKAN20R)	No
5	mka sp. primers with mka genome (CMYC20F + SKAN20R)	114
6	Common primers with mka genome (CMYC20F + CMYC518R)	143

IV. DISCUSSION

In our study, focus was made on the methodology development which involved 16S rRNA based PCR amplification study for early diagnosis of *Mycobacterium tuberculosis, Mycobacterium smegmatis and Mycobacterium kansasii,* where msm and mka belongs to MOTT family. By involving e-PCR (Primer BLAST) specific primers were designed for 16S locus of all three species along with one universal primer set which can detect all three species. Use

of 16S ribosomal DNA for PCR based diagnosis was recommended for many instances and registered a remarkable sensitivity and success especially with clinical samples [2], [14]-[17]. According to results, primer combination of CMYC20F with SMTB18R has given the specific amplification of 209 bp with mtb genome. While, primers combination of CMYC20F with SKAN20R shown the amplicons of 114 bp with mka genome and CMYC20F with SMSM19R shown the amplicon of 106 bp with msm genome. In decision, these three primer sets were nominated as marker primers for unambiguous identification of mtb, mka and msm respectively. In contrast when species specific primers mixed with non specific genomes, amplifications were not recorded this further ensured its specificity and sensitivity.

Along with this, a universal primer set has been tested and they possessed the ability to detect and amplified 16S regions of all three mycobacterium species and hence could be considered as positive control for previous three primer sets. When sequences of amplicons obtained from CMYC20F + CMYC518R along with mtb, msm and mka genome, it was found of 149 bp, 149 bp and 143 bp respectively.

Based on the results of 16S PCR and sequencing it could be concluded that, the PCR based diagnosis of the mtb, mka and msm could be possible in the patient samples by using specified primer sets and defined PCR conditions. In order to gain early detection other mycobacterium species, similar methodology could be adopted. This step in treatment procedure requires higher specificity and sensitivity in order to prescribe proper therapy to the patients. Our work will provide a platform for early detection of MOTT against TB infection and certainly a cheaper method developed for DNA isolation and PCR will lower down the cost of the diagnosis.

REFERENCES

[1] B. R. Bloom,, and C. J. L. Murray, "Tuberculosis: commentary on a re-emergent killer." *Science*, 1992, 257:1055–1064.

[2] B. Bo¨ddinghaus, T. Rogall, T. Flohr, H. Blo¨cker, and E. C. Bo¨ttger, "Detection and identification of mycobacteria by amplification of rRNA". *J.Clin. Microbiol*. 1990, 28:1751–1759.

[3] J. J. Ellner, A. R. Hinman, S. W. Dooley, M. A. Fischl, K. A. Sepkowitz, M. J. Goldberger, T. M. Shinnick, M. D. Iseman, and W. R. Jacobs, Jr., "Tuberculosis symposium: emerging problems and promise." *J. Infect. Dis*., 1993, 168:537–551.

[4] J. Malkin, A. Shrimpton, M. Wiselka, M.R. Barer, M. Duddridge, N. Perera, "Olecranon bursitis secondary to *Mycobacterium kansasii* infection in a patient receiving infliximab for Behcet's disease." *J Med Microbiol.* 2009 ,Mar, 58(Pt 3):371-3.

[5] R. W. Shafer, and M. F. Sierra, "*Mycobacterium xenopi, Mycobacterium fortuitum, Mycobacterium kansasii*, and other nontuberculous mycobacterium in an area of endemicity for AIDS." *J. Clin. Infect. Dis.*, 1992, 15:161–162.

[6] P. A. Catherine, J. Emmanuelle, L. Salma, A.J. R. Frédéric, V. Véronique, C.Danielle, E. Jean-François, F. Alain, B. Stéphane, G. Jean-Louis, and C. Jean-Laurent,"Fatal Disseminated *Mycobacterium smegmatis* Infection in a Child with Inherited Interferon? Receptor Deficiency". *Clin. Infect.Dis*. ,1997, 24 (5): 982-984.

[7] E.D. Manali, W.J. Tomford, D.W. Liao, C. Farver, A.C. Mehta, "*Mycobacterium kansasii* endobronchial ulcer in a non immune-compromised patient". *Respiration*, 2005, May-Jun,72(3):305-8.

[8] W. T. Miller, Jr., and W. T. Miller, Sr., "Pulmonary infections with atypical mycobacterium in the normal host." *Semin. Roentgenol*.,1993, 28:139–149.

[9] H.A. Reimann, "MICROCOCCUS TETRAGENUS INFECTION: I. "Review of the Literature, Report of a Non-fatal Case with Septicemia, Meningitis and Arthritis, and Bacteriologic Studies." *J Clin Invest*., 1935, May,14(3):311-9.

[10] G.A. Dascalopoulos, S. Loukas, S.H. Constantopoulos, "Wide geographic variations of sensitivity to MOTT sensitins in Greece". *Eur Respir J.*, 1995, May,8(5):715-7.

[11] E.M. Salvana, G.S. Cooper, R.A. Salata, "Mycobacterium other than tuberculosis (MOTT) infection: an emerging disease in infliximab-treated patients." *J Infect*., 2007, Dec,55(6):484-7.

[12] G.L. Woods, J.A. Washington 2nd, "Mycobacteria other than Mycobacterium tuberculosis: review of microbiologic and clinical aspects". *Rev Infect Dis*. ,1987, Mar-Apr, 9(2):275-94.

[13] K.P.Cain, K.D. McCarthy, C.M. Heilig, P. Monkongdee, T .Tasaneeyapan, N. Kanara, M.E. Kimerling, P. Chheng, S. Thai, B. Sar, P. Phanuphak, N. Teeratakulpisarn, N. Phanuphak, H.D. Nguyen, T.Q .Hoang, H.T. Le, J.K. Varma, "An algorithm for tuberculosis screening and diagnosis in people with HIV". *N Engl J Med.*, 2010, Feb 25,362(8):707-16.

[14] M. S., Hughes, R. A. Skuce, L. A. Beck, and S. D. Neil, "Identification of mycobacteria from animals by restriction enzyme analysis and direct DNA cycle sequencing of polymerase chain reaction-amplified 16S rRNA gene sequences." *J. Clin. Microbiol*. , 1993, 31:3216–3222.

[15] P. Kirschner, B. Springer, U. Vogel, A. Meier, A. Wrede, M. Kiekenbeck,F. C. Bange, and E. C. Bo¨ttger, "Genotypic identification of mycobacteria by nucleic acid sequence determination: report of a 2-year experience in a clinical laboratory., 1993.

[16] B. B.Plikaytis, B. D. Plikaytis, M. A. Yakrus, W. R. Butler, C. L. Woodley,V. A. Silcox, and T. M. Shinnick., "Differentiation of slowly growing Mycobacterium species, including Mycobacterium tuberculosis, by gene amplification and restriction fragment length polymorphism analysis." *J. Clin.Microbiol*., 1992, 30:1815–1822.

[17] M. Vaneechoutte, H. de Beenhouwer, G. Claeys, G. Verschraegen, A. de Rouck, N. Paepe, A. Alachouni, and F. Portaels, "Identification of Mycobacterium species by using amplified ribosomal DNA restriction analysis." *J. Clin. Microbiol*., 1993, 31:2061–2065.

Dilip G. Gore was born in Nagpur, India and pursuing PhD in the sericulture. Dilip Gore received M.Sc. Degree in Biotechnology in 2006 from Nagpur University, India. Currently he is lecturer in Biotechnology and Microbiology department of G. H. Raisoni institute of Interdisciplinary Sciences nagpur. He is research head for the Sai Bioinfosys Bioinformatics research centre. He works in the area of drug designing, Vaccine development and invertebrate pathology along with bioinformatics as specialization. He chaired number of national and international conferences of bioinformatics with special talk on bacterial genomics and functionality search. He is an eminent speaker for the computational biology and genomics. He got seven international publications to his name in the area of genomics and vaccinology. He is faculty member of yashwantrao chavan open university for the examination section.

Niharika M. Amrute was born in Betul, India and received M.Sc. degree in Biotechnology in 2011 awarded by Nagpur University. Currently she is working as a lecturer in Vivekanand vigyan mahavidyalaya and stand in top rank in the exams of M.Sc. biotechnology conducted by Nagpur University. She was a student of Biotechnology department of G.H. Raisoni institute of Interdisciplinary Sciences Nagpur.

Atul P. Denge was born in Nagpur, India and received M.Sc. degree in Biotechnology in 2011 awarded by Nagpur University. Currently he is working as research associate in Sai Bioinfosys and stand in top rank in the exams of M.Sc. biotechnology conducted by Nagpur University. He was a student of Biotechnology department of G.H. Raisoni institute of Interdisciplinary Sciences Nagpur.

Dr. Suchitra S. Godbole was born in Nagpur, India and Received M.Sc. and PhD degree in Microbiology awarded by Nagpur University. She is a head of the department of G.H. Raisoni institute of Interdisciplinary Sciences Nagpur. She worked in the field of environmental management and having number of publications and patent to her name. She chaired number of conferences and judged several biological quiz competitions on national level. She is a faculty member of yashwantrao chavan open university and Nagpur University and PhD guide for Microbiology.