Method

Development and Evaluation of a Multiplex PCR for Rapid Detection and Differentiation of Mycobacterium tuberculosis Complex Members from Non-Tuberculous Mycobacteria

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SUMMARY: Most mycobacterial infections are still caused by Mycobacterium tuberculosis complex (MTC) strains; however, infections by non-tuberculous mycobacteria (NTM) are increasing, particularly among immunocompromised patients. Conventional species-specific identification and proper patient management are delayed due to the slow-growing nature of mycobacteria. We have developed a multiplex PCR (mPCR) targeting the oxyR-ahpC intergenic region and rpoB gene for direct detection and differentiation of clinical isolates as MTC or NTM in primary culture. Two amplicons of 473 bp and 235 bp from MTC members and a single amplicon of 136 bp from NTM are expected. The mPCR was developed using several mycobacterial species and was evaluated by testing extracted DNA from liquid cultures, flagged as positive for bacterial growth, of 100 consecutive mycobacterial isolates. The results were validated by DNA sequencing of the species-specific 16S-23S internal transcribed spacer (ITS) region. The mPCR with template DNA from reference Mycobacterium spp. yielded the expected amplicons. When 100 consecutive clinical isolates of Mycobacterium spp. were tested, 92 strains yielded MTC member-specific amplicons, and DNA sequences from 10 randomly selected isolates matched completely with the ITS sequence from M. tuberculosis. Eight isolates were identified as NTM, and DNA sequencing of the ITS region confirmed the NTM status of each of these isolates. The mPCR developed in this study allowed rapid detection and differentiation of primary cultures as MTC or NTM, thus helping in timely institution of specific therapy.

INTRODUCTION

Tuberculosis (TB), with nearly 8 million cases of active disease and two million deaths occurring annually, is a major public health problem around the world (1). With the continued expansion of human immunodeficiency virus (HIV) infection and growing population of other immunocompromised patients, infections with non-tuberculous mycobacteria (NTM) as well as TB have increased in many parts of the world (1-3). While TB due to Mycobacterium tuberculosis complex (MTC) strains is the most common mycobacterial infection in developing countries, many NTM are also of medical relevance, particularly for immunocompromised patients (2,4). The NTM infections occur more frequently in developed countries where the incidence of TB is low. Specific diagnostic methods for rapid detection and differentiation of NTM infections from TB during early stages of diagnosis are urgently needed, since many NTM are resistant to the antibiotics used for treatment of TB (1,2,4). The traditional diagnosis of mycobacterial infections based on culture and phenotypic identification is time consuming. Primary culture of slowly growing mycobacteria on solid media usually takes 4-6 weeks and it is sometimes difficult to discriminate among closely related species (5). Although molecular methods have not been able to replace culture for the detection of mycobacteria in clinical specimens, their application combined with cultivation has accelerated the laboratory diagnosis of mycobacterial infections (6-8). The new generation of liquid cultures is rapid (10-12 days) and the mycobacterial growth obtained in these cultures has been successfully used for reliable detection of MTC or NTM both by conventional and molecular methods (5,9).

Recently, PCR or PCR-linked methods have been used for rapid detection and differentiation of MTC and NTM. Multiplex PCR (mPCR) targeting of many different genes simultaneously has been used to detect and identify MTC and NTM in routine diagnostic laboratories (10-12). However, some of these methods yield false-negative results, as the target sequences (such as IS6110) are not uniformly present in all clinical isolates. Although the development of DNA probes has greatly improved mycobacterial identification, particularly MTC, the commercially available AccuProbe DNA probe system (Gen-Probe, San Diego, Calif., USA), besides being expensive, offers only a limited number of species-specific probes and the clinical isolates are mostly identified as MTC or NTM (13,14). The PCR-based reverse hybridization line probe assays (INNO-LiPA Mycobacteria and Genotype Mycobacteria) (15,16) are expensive, and their complex patterns make it difficult to implement them in a routine diagnostic laboratory. The DNA sequencing of mycobacterial gene targets such as 16S rRNA (17), rpoB (18), hsp65 (19), secA (20) and 16S-23S internal transcribed spacer (ITS) region (21) genes have the potential for species-specific identification of nearly all Mycobacterium spp. However, DNA sequencing of all culture isolates in a clinical diagnostic laboratory is not practical due to its prohibitive cost, particularly in resource-poor developing countries. Since TB is the most common mycobacterial infection in develop-

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ing countries (1), a simple, inexpensive and reliable method for discriminating MTC from NTM that can be applied immediately to cultures flagged as positive in liquid media (such as a mycobacterial growth indicator tube [MGIT] 960 system) is highly desirable. The minority of NTM could then be identified to the species level by more sophisticated and highly discriminatory methods such as DNA sequencing of the ITS region.

The aim of this study was to develop a simple and robust mPCR for rapid detection and differentiation of MTC from NTM directly from liquid cultures flagged positive for bacterial growth. The results were then validated by testing 100 consecutive clinical isolates of Mycobacterium spp. in Kuwait.

MATERIALS AND METHODS

Bacterial species and strains: The reference strains or well characterized clinical isolates of several Mycobacterium spp. were used and included M. tuberculosis, M. bovis BCG, M. avium, M. intracellulare, M. kansasi, M. fortuitum, M. gastri, M. phlei, M. chelonae, M. abscessus, M. gordonae, M. scrofulaceum, M. chimaera, M. immunogenenum and M. smegmatis. To evaluate whether amplification in mPCR was specific for DNA originating from Mycobacterium spp., genomic DNA from several commonly encountered bacterial or fungal species such as Escherichia coli, Salmonella typhimurium, Campylobacter jejuni, Helicobacter pylori, Staphylococcus aureus, Candida albicans and Aspergillus fumigatus was also used. One hundred consecutive clinical isolates of Mycobacterium spp. recovered from various clinical specimens at the Kuwait National Central Laboratory for the diagnosis of TB were analyzed to evaluate mPCR.

Template DNA preparation: The reference Mycobacterium spp. were grown as liquid cultures using the MGIT 960 system by following the standard procedures and as recommended by the manufacturer of the MGIT system (Becton Dickinson, Sparks, Md., USA) (22). The genomic DNA from reference Mycobacterium spp. was isolated as described in detail previously (23). The conditions for growth and isolation of genomic DNA from other bacterial or fungal organisms are also described in detail elsewhere (24). The isolation of Mycobacterium spp. from various clinical specimens was also performed by using the MGIT 960 system, and the genomic DNA from culture tubes just flagged positive for growth was prepared by the boiling method to reduce the DNA preparation time and by incorporating the removal of PCR inhibitors as follows. One milliliter of mycobacterial culture was heated with 40 mg of Chelex-100 (Sigma, St. Louis, Mo., USA) at 95°C for 10 min and the supernatant obtained following centrifugation at 12,000 × g for 15 min was used as the source of genomic DNA for amplification in mPCR.

Development of mPCR: The oxyR and ahpC genes are divergently transcribed in mycobacteria. M. tuberculosis and other members of MTC naturally contain a defective oxyR due to mutations in the 5′ end of the gene while NTM contain a functional gene copy (25). Thus, PCR amplification with MTC-specific primers IGRF (5′-AGCGTCTGGTCGCGT AAGCAGTG-3′) and IGRR (5′-GGTGGAAGTAGTCGCC GGGCTGCT-3′) should yield a 473-bp fragment from M. tuberculosis and other MTC members, while no amplification should occur from NTM. The rpoB gene among Mycobacterium spp. also contains variable sequences that have previously been exploited for identification and differentiation of MTC from NTM (12,18). Since the previously described MTC-specific primer sequences derived from the rpoB gene (12) differ from the DNA sequences of the corresponding region from several NTM species by a single nucleotide at the 3′ end, we have slightly modified these primer sequences. The MTC-specific primer sequences at the 3′ end were extended by an additional nucleotide for greater specificity and also to avoid the effect of n-1 primers that may be generated during synthesis. Thus, a variable rpoB gene region from MTC or NTM was amplified using MTC-specific (MTCF, 5′-TACGGTGCCGACGTGACAAA-3′ and MTCR, 5′-ACAGTCGGGCTTGGTTCAAC-3′) or NTM-specific (NTMF, 5′-GGAGCGGATGACCACCCCG GACGTC-3′ and NTMR, 5′-CAGGCGTTGTGTCTGGTC CATGAAC-3′) primers, and amplicons of 235 bp and 136 bp are expected from MTC and NTM, respectively. The uniplex or multiplex PCR was performed under the conditions developed recently in our laboratory for efficient amplification of mycobacterial DNA (26). Briefly, the reaction mixtures in a final volume of 50 μl contained Perkin-Elmer PCR buffer, 10 pmol of either IGRF and IGRR or MTCF and MTCR or NTMF and NTMR (for uniplex PCR) or all six primers simultaneously (for mPCR). 2 μl of template DNA, 0.1 mM dNTP and 2 units of AmpliTaq DNA polymerase. The cycling parameters included an initial denaturation at 95°C for 3 min and 97°C for 1 min; 2 cycles of 1 min at 95°C, 30 s at 64°C, and 1 min at 72°C; 2 cycles of 1 min at 95°C, 30 s at 62°C, and 1 min at 72°C; 2 cycles of 1 min at 95°C, 30 s at 60°C, and 1 min at 72°C; 2 cycles of 1 min at 95°C, 30 s at 58°C, and 1 min at 72°C; 22 cycles of 1 min at 95°C, 30 s at 56°C, and 1 min at 72°C; followed by an additional cycle of 10 min at 72°C. The reactions were started by the addition of dNTPs (hot start) (26). The amplified products were analyzed by electrophoresis in 2% agarose gels, performed as described previously (24).

Evaluation of mPCR: One hundred consecutive Mycobacterium spp. isolated from various clinical specimens in Kuwait were tested to evaluate the performance of mPCR. The isolates were mostly recovered from active pulmonary/ extrapulmonary TB patients but were also obtained from patients suspected of having other mycobacterial infections. The identification of clinical isolates was also carried out by conventional as well as by AccuProbe methods (5,14). The AccuProbe assay was performed on MGIT system tubes within the first working day following instrument detection of growth as directed by the supplier of the kit and as described previously (14). Briefly, a 1.5-ml portion of liquid medium was centrifuged for 10 min in a sterile screw cap microcentrifuge tube, and the pellet was resuspended in 0.1 ml of the lysis reagent and then tested against the DNA probes by using the AccuProbe protocol according to the manufacturer’s instructions. Samples producing signals greater than 30,000 relative light units were considered as true positives. The mPCR was also performed on DNA extracted from liquid cultures flagged as positive for bacterial growth in the MGIT 960 system to demonstrate its utility in early detection and identification of clinical Mycobacterium spp.

Confirmation of mPCR results by DNA sequencing of the 16S-23S ITS region: The results of mPCR for some randomly selected MTC isolates and all NTM strains were validated by PCR amplification of the 16S-23S ITS region followed by direct DNA sequencing of the amplified DNA. The ITS region was amplified by using pan-mycobacterial primers MYITSF (5′-GATTGGGACGAAGTCGTAACA-3′ and
tested by growing mycobacterial DNA released from only a few cells. This was a protocol that was highly sensitive and should be able to detect to 4,000 tubercle bacilli. This suggested that the established tuberculosis methods as well as by AccuProbe. However, of the eight isolates also identified as MTC members by the conventional methods, eight isolates were identified as NTM (Table 1). All the 92 strains yielded MTC member-specific amplicons while the data from seven isolates are shown in Figure 2. In mPCR, used for their identification as MTC or NTM by mPCR, and cultures flagged as positive by the MGIT 960 system was Kuwait. The DNA extracted directly from mycobacterial bacterium (data not shown).

The lower limit of sensitivity of mPCR was determined by serially diluting genomic DNA (20 ng to 2 fg) of M. tuberculosis. The mPCR was positive with 20 pg of M. tuberculosis DNA (data not shown), corresponding roughly to 4,000 tubercle bacilli. This suggested that the established protocol was highly sensitive and should be able to detect mycobacterial DNA released from only a few cells. This was tested by growing M. tuberculosis in an MGIT 960 system and by extracting DNA from MGIT system tubes flagged as positive for bacterial growth. The mPCR yielded MTC-specific amplicons of ~473 bp and ~235 bp from each MGIT system tube that was flagged as positive for bacterial growth (data not shown).

The mPCR was then applied to 100 consecutive Mycobacterium spp. isolated from various clinical specimens in Kuwait. The DNA extracted directly from mycobacterial cultures flagged as positive by the MGIT 960 system was used for their identification as MTC or NTM by mPCR, and the data from seven isolates are shown in Figure 2. In mPCR, 92 strains yielded MTC member-specific amplicons while eight isolates were identified as NTM (Table 1). All the 92 mycobacterial isolates identified as MTC by mPCR were also identified as MTC members by the conventional methods as well as by AccuProbe. However, of the eight isolates identified as NTM by mPCR, one isolate each was identified as M. avium and M. intracellulare, while the remaining six isolates were identified as NTM only by AccuProbe. Direct DNA sequencing of the 16S-23S ITS region from 10 randomly selected MTC isolates matched completely with the

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<th>No. of isolates</th>
<th>mPCR</th>
<th>AccuProbe</th>
<th>ITS sequencing</th>
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<tr>
<td>10 MTC</td>
<td>MTC</td>
<td>M. tuberculosis</td>
<td></td>
</tr>
<tr>
<td>82 MTC</td>
<td>MTC</td>
<td>Not done</td>
<td></td>
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<tr>
<td>1 NTM M. avium</td>
<td>M. avium</td>
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<tr>
<td>1 NTM M. intracellulare</td>
<td>M. intracellulare</td>
<td></td>
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<tr>
<td>2 NTM NTM</td>
<td>M. abscessus</td>
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<tr>
<td>2 NTM</td>
<td>M. fortuitum</td>
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<td>1 NTM NTM</td>
<td>M. malmoense</td>
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<td>1 NTM</td>
<td>M. scrofulaceum</td>
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MTC, Mycobacterium tuberculosis complex; NTM, non-tuberculous mycobacteria.
ITS sequence from M. tuberculosis (Table 1). Further, DNA sequencing of the ITS region led to species-specific identification of all the eight isolates that were identified as NTM by mPCR (Table 1).

DISCUSSION

The incidence of TB varies considerably around the world, and most mycobacterial infections in the developing countries are still caused by MTC members (1). Although Kuwait, with nearly 25 cases per 100,000 population, has a relatively low TB incidence among countries of the Arabian Gulf region, it also has a large expatriate work force originating from TB endemic countries of South Asia (such as India, Pakistan and Bangladesh), Southeast Asia (mainly the Philippines and Indonesia) and the Middle East (such as Egypt) (23,27). About 600 patients, nearly 75% being expatriates, are diagnosed with mycobacterial infections every year, and M. tuberculosis is the most frequently isolated species, accounting for nearly 90% of all mycobacterial infections in Kuwait (23,27). The cultivation of Mycobacterium spp. is carried out by solid and liquid cultures (using an MGIT 960 system) followed by their identification by conventional methods (5). Recently, AccuProbe was introduced for rapid species-specific identification of all Mycobacterium spp. isolates in Kuwait. However, since the majority of mycobacterial infections are caused by M. tuberculosis and the AccuProbe system, besides being expensive, offers only a few probes for species-specific identification of NTM (14), most of the isolates are either identified as MTC or NTM in Kuwait. Rapid and early differentiation of MTC members from NTM and species-specific identification of all NTM are crucial for proper patient management, since many NTM are resistant to antibiotics commonly used for the treatment of mycobacterial infections (2,4).

In this study we have developed a sensitive and specific mPCR using the oxyR-ahpC intergenic region and the variable region of the rpoB gene as targets for rapid differentiation of MTC from NTM directly from primary liquid cultures flagged positive for bacterial growth. All MTC members (M. tuberculosis, M. bovis, M. africanum and M. microti) isolates are natural mutants for the oxyR gene due to several deletions/other mutations, while NTM contain a functional gene copy (25). Thus, PCR amplification with the primers designed in this study yielded an amplicon of 473 bp only from MTC and not from NTM, as expected. Although variable rpoB gene sequences have previously been used in a duplex PCR for differentiation of MTC from NTM (12), we have slightly modified these primer sequences for greater specificity for the following reasons. The MTC-specific forward and reverse primer sequences described previously (12) vary by only one or two nucleotides near the 3′-end from most Mycobacterium spp. However, rpoB gene sequences corresponding to the 3′-end of the forward primer (corresponding to nt 4-23 of primer MTCF described above) from one clinical isolate of M. kansasii and a few isolates of M. capri and M. xenopi are completely identical. Since rpoB is an essential gene that is uniformly present in all mycobacterial species, the chance occurrence of nearly identical sequences in the rpoB gene corresponding to MTC-specific primers may result in misidentification of some NTM strains as MTC. Secondly, generation of n-1 primers (during synthesis or due to storage and repeated freezing and thawing) will result in the loss of MTC specificity of the MTCF and MTCR primers. The above scenario also necessitated the inclusion of MTC-specific oxyR-ahpC intergenic region in a multiplex format for greater specificity and to avoid misidentification of some NTM strains as MTC members. All MTC members yielded two amplicons of 473 bp and 235 bp while NTM yielded a single amplicon of 136 bp in mPCR. Thus, the mPCR developed in this study was highly specific with easily interpretable results. Although other investigators have also described mPCR assays for differentiation of MTC from NTM (10,11,28), some of the genes used as targets are either not specific for M. tuberculosis or they may yield false-negative results. For example, the IS6110 used as a target in nearly all mPCR assays developed previously (10,11,28) may yield false negative results from a sizeable number of specimens from some geographical locations since the copy number of IS6110 in M. tuberculosis isolates around the world varies from 25 to 0 (29). The mPCR developed in this study was also quite sensitive, as it detected M. tuberculosis DNA released from nearly 4,000 tubercle bacilli as well as from MGIT 960 system tubes flagged as positive for bacterial growth.

The usefulness of the mPCR assay was evident from the fact that when 100 consecutive clinical isolates of Mycobacterium spp. in Kuwait were analyzed, it identified 92 isolates as MTC and eight isolates as NTM. These results were totally in agreement with those obtained with the AccuProbe DNA probe assay. Although the latter method identified two NTM to the species level, the majority (six of eight) were only identified as NTM. Since the majority of mycobacterial infections in developing countries are still caused by MTC, the AccuProbe DNA probe assay offers only a small benefit over the mPCR assay developed in this study, if applied to all mycobacterial isolates due to its prohibitive cost for most of the mycobacteriology laboratories of the developing countries. The application of other PCR-based methods, such as reverse hybridization with species-specific probes (15,16) and direct DNA sequencing of variable regions of mycobacterial genomes (18-21), is also not practical on all culture isolates of Mycobacterium spp. as these methods are expensive and/or technically demanding.

In conclusion, our laboratory has developed a sensitive and specific mPCR assay for rapid detection and differentiation of Mycobacterium spp. isolates as MTC or NTM directly in primary culture. The small minority of NTM isolates in TB endemic countries could then be identified to the species level by direct DNA sequencing of the highly variable and species-specific 16S-23S ITS region. To the best of our knowledge, this is the first use of the oxyR-ahpC intergenic region and the rpoB gene for developing an mPCR for rapid detection and differentiation of MTC from NTM.

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