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Novel Multiplex PCR Using Dual-Priming Oligonucleotides for Detection and Discrimination of the *Mycobacterium tuberculosis* Complex and *M. bovis* BCG▽

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We developed a novel multiplex PCR assay using dual-priming oligonucleotide primers targeting the *RD1* gene for simultaneous identification of the *Mycobacterium tuberculosis* complex and *M. bovis* bacillus Calmette-Guérin (BCG). This assay would be useful both for detection of the *M. tuberculosis* complex and for differentiation of *M. bovis* BCG from pathogenic *M. tuberculosis* complex species.

Tuberculosis is a major global public health problem. This disease, mainly caused by the *Mycobacterium tuberculosis* complex, is still found in areas of endemicity in developing countries and has reemerged in developed countries with the increase in the incidence of AIDS and other immunocompromising conditions (6). In South Korea, 95 to 99% of children are vaccinated with *M. bovis* bacillus Calmette-Guérin (BCG) (12). However, complications have been reported, including *M. bovis* BCG dissemination, osteomyelitis, abscesses, and lymphadenitis (5). In addition, *M. bovis* BCG has been used for the treatment of bladder cancer. For these patients, complications that have included pneumonitis, hepatitis, and noncaseating granulomas have also been reported (9). It has been reported that susceptibilities to antituberculosis drugs differ between *M. tuberculosis* and *M. bovis* BCG (17). Therefore, it is important for clinicians to distinguish *M. bovis* BCG from other members of the *M. tuberculosis* complex, especially for patients who have a history of *M. bovis* BCG vaccination and treatment.

In this study, to develop a novel multiplex PCR assay for the detection and discrimination of the *M. tuberculosis* complex and *M. bovis* BCG, the RD1 sequence, which is absent in *M. bovis* BCG but is present in the *M. tuberculosis* complex (12, 14), was selected for analysis using dual-priming oligonucleotide (DPO) primers (4).

To validate the novel multiplex PCR assay, we tested the following isolates: (i) reference strain isolates from the American Type Culture Collection (ATCC), the Korean Type Culture Collection (KTCC), and the Korean Institute of Tuberculosis (KIT) of the Korean National Tuberculosis Association, including two *M. tuberculosis* isolates (ATCC 25177 and ATCC 27294), 13 isolates representing nontuberculous mycobacteria (NTM; *M. abscessus* ATCC 19977, *M. avium* ATCC 25291, *M. fortuitum* KTCC 1122, *M. intracellulare* KIT 41105, *M. kansasii* KTCC 9515, *M. margeritense* ATCC 700351, *M. marinum* ATCC 927, *M. mucogenicum* KTCC 19088, *M. nonchromogenicum* ATCC 19530, *M. peregrinum* KTCC 9615, *M. scrofulaceum* KTCC 9519, *M. septicum* ATCC 700731, and *M. szulgai* KTCC 9520), and four isolates representing nonmycobacterial species (*Escherichia coli* ATCC 25922, *Haemophilus influenzae* ATCC 9007, *Klebsiella pneumoniae* ATCC 700603, and *Staphylococcus aureus* ATCC 29213); (ii) isolates from two patients with *M. bovis* BCG infections (BCG-oma or BCG osteitis) previously proven using in-house, real-time PCR with two hydrolysis probes (3); and (iii) extracts from two commercial *M. bovis* BCG vaccines, the Tokyo 172 BCG vaccine (Japan BCG Manufacturing Corporation, Tokyo, Japan) and the Pasteur 1173p2 vaccine (Korean National Tuberculosis Association, Seoul, South Korea).

DNA was extracted from the reference strain isolates and *M. bovis* BCG by a heating method; each colony was isolated from culture media and suspended in 500 μl of 1× Tris-EDTA buffer. The solution was heated to 100°C for 20 min, and after centrifugation at 15,000 rpm for 5 min, the supernatant was used for PCR. DNA was extracted from commercial BCG vaccines by the use of a QIAamp blood Mini kit (Qiagen, Hilden, Germany) or a viral RNA kit (Qiagen).

The four pairs of DPO primers for amplification of the *RD1* gene were designed to generate amplicons of different sizes from the *M. bovis* BCG genome or from the *M. tuberculosis* complex (Fig. 1). The BCG-F and RD1-R3 primers, which are complementary to sequences flanking the deleted region, produced a 364-bp PCR amplicon from *M. bovis* BCG strains from which the *RD1* region had been deleted. No PCR product was generated from the *M. tuberculosis* complex by this pair of primers, because the two primers are too far apart to amplify efficiently the entire 9,557-bp region in the *M. tuberculosis* complex. In mycobacterial species with the *RD1* region present in the genome, a copy of the 208-bp product was produced by primers RD1-F3 (which is complementary to the *RD1* sequence) and RD1-R3. Two copies of the amplicons were also generated using primer pair RD1-F1 and RD1-R1 and primer pair RD1-F2 and RD1-R2; these amplicons are complemen-

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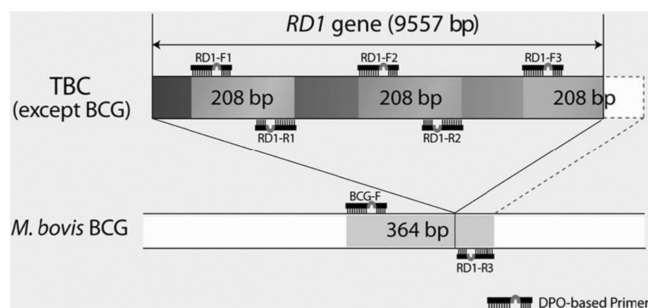

FIG. 1. Design of a novel multiplex PCR: a schematic representation of the *RD1* gene and of the locations of the novel dual-priming oligonucleotide (DPO) primers and their PCR amplicons. TBC, *M. tuberculosis* complex.

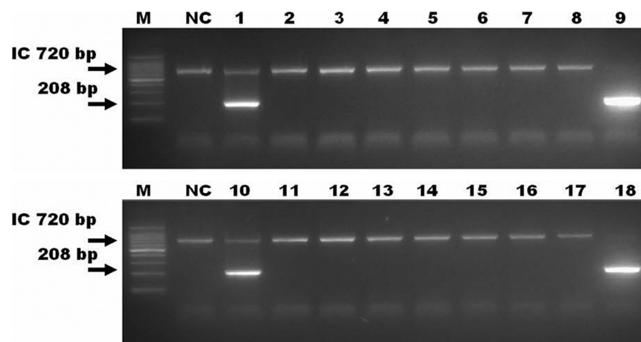

FIG. 2. The specificity of the novel assay using species of nontuberculous mycobacteria (NTM). Each lane contains a PCR product amplified from DNA of one of the following mycobacterial species: *M. tuberculosis* (lanes 1, 9, 10, and 18), *M. avium* (lane 2), *M. abscessus* (lanes 3 and 4), *M. intracellulare* (lane 5), *M. fortuitum* (lane 6), *M. kansasii* (lane 7), *M. mucogenicum* (lane 8), *M. peregrinum* (lane 11), *M. scrofulaceum* (lane 12), *M. septicum* (lane 13), *M. marinum* (lane 14), *M. szulgai* (lane 15), *M. margeritense* (lane 16), and *M. nonchromogenicum* (lane 17). Lane NC represents a negative control, and lane M contains molecular size markers (100-bp ladder). The expected sizes of the PCR products are marked on the left, and the 720-bp internal control (IC) amplicon was detected in each test sample.

tary to different sequences within *RD1* but amplify regions that are all the same size (208 bp). To confirm proper amplification, this multiplex PCR included amplification of the *cellulose synthase 3* gene from *Arabidopsis thaliana*, which generates a 720-bp internal control band. PCR was performed using a 20-μl reaction mixture containing 1× Master Mix, 1× primer mix, and 3 μl of DNA. All cycling profiles incorporated an initial denaturation at 94°C for 15 min; 40 amplification cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C; a final extension at 72°C for 5 min; and then a hold at 4°C. PCR products were visualized by 2.0% agarose gel electrophoresis and ethidium bromide staining. To reduce the risk of contamination and carryover, we observed universal standard precautions for nucleic acid amplification and included negative controls in each run.

Our novel assay with DPO primers reliably produced 208-bp products from the *M. tuberculosis* complex samples, and no false positives were seen with the four nonmycobacterial species (data not shown). The specificity of the novel multiplex assay was also determined using NTM isolates that are frequently found in human specimens. Among the isolates from 13 reference strains of NTM, no isolate produced a 208-bp PCR product from *RD1* (Fig. 2). To evaluate the discriminatory power of the novel multiplex PCR, the following samples were tested and the results compared: clinical isolates from patients diagnosed with BCG-oma or BCG osteitis (12); extracts from the *M. bovis* BCG vaccines BCG Pasteur 1173p2 and BCG Tokyo 172; *M. tuberculosis* isolates; and NTM isolates (*M. intracellulare*, *M. avium*, and *M. abscessus*). In contrast to the 208-bp amplicons produced from the *M. tuberculosis* complex isolates, a single 364-bp amplicon was observed in all specimens of *M. bovis* BCG. In samples containing DNA from NTM, neither the 364-bp nor the 208-bp product was observed after amplification (Fig. 3).

Confirmation of *M. bovis* BCG infection is difficult with molecular tests. 16S rRNA sequences have been used for the identification of bacteria other than the *M. tuberculosis* complex, but these sequences are highly homologous among *M. tuberculosis* complex species. Thus, this sequence is not suitable for differentiating *M. bovis* BCG from *M. tuberculosis* complex species (10). Another frequently targeted sequence used to detect the *M. tuberculosis* complex is the insertion sequence IS*6110*, which is present in the *M. tuberculosis* complex genome in various copy numbers (7). However, the use of IS*6110* for the detection and differentiation of the *M. tuberculosis* complex and *M. bovis* BCG has some limitations. The use of IS*6110* alone cannot differentiate *M. bovis* BCG (1 to 2 copies of IS6110) from *M. bovis* (1 to 6 copies) (15). Moreover, the copy numbers of IS*6110* are variable among species within the *M. tuberculosis* complex (8, 11, 18, 19, 20). Therefore, alternative targets have been investigated for the detection and differentiation of *M. bovis* BCG and the *M. tuberculosis* complex.

In this study, the *RD1* gene was selected as the target of the novel assay to detect and discriminate the *M. tuberculosis* complex and *M. bovis* BCG by the use of single-round amplification. Previous studies of *M. bovis* BCG substrains have elucidated more than 100 genomic regions that are absent in *M. bovis* BCG but present in *M. tuberculosis* (2, 13). These deleted sequences are referred to as regions of difference (RD), and

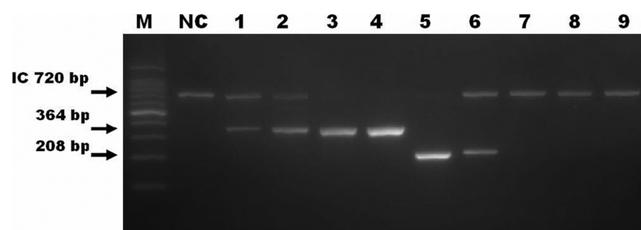

FIG. 3. Detection and discrimination of *M. tuberculosis* complex species and *M. bovis* bacillus Calmette-Guérin (BCG) by the use of a novel assay. Each lane contains a PCR product amplified from DNA of the following mycobacteria: BCG Pasteur, isolated from a patient (lane 1); BCG Tokyo, isolated from a patient (lane 2); BCG Pasteur 1173p2, vaccine (lane 3); BCG Tokyo 172, vaccine (lane 4); and isolates of *M. tuberculosis* (lanes 5 and 6), *M. intracellulare* (lane 7), *M. avium* (lane 8), and *M. abscessus* (lane 9). Lane NC represents a negative control, and lane M contains molecular size markers (100-bp ladder). The expected sizes of the PCR products are marked on the left, and the 720-bp internal control (IC) amplicon was detected in each test sample.

RD1 is absent from all BCG substrains and from the related live vaccine strain of *M. microti* but is present in virulent *M. bovis* and *M. tuberculosis* (2, 14). The *RD1* region is not shared by most nontuberculous species (1, 16). Therefore, we developed a multiplex PCR for the detection and discrimination of *M. tuberculosis* and *M. bovis* BCG based on amplification of the *RD1* sequence and the use of DPO primers. A DPO contains two different priming regions (a stabilizer and a determiner) joined with polydeoxyinosine. The polydeoxyinosine linker prevents formation of secondary structures and effectively eliminates nonspecific priming, thus decreasing nonspecific products in the PCR. The linker enables the more specific detection of the target pathogen (4). DPO in conjunction with amplification of the *RD1* sequence is a novel tool for the molecular discrimination of highly homologous mycobacterium species.

In conclusion, our novel multiplex PCR is a sensitive and reliable assay for differential diagnosis of tuberculosis. This novel assay would be useful in clinical situations of suspected tuberculosis, not only for the rapid detection of the *M. tuberculosis* complex in clinical specimens but also for the differentiation of *M. bovis* BCG from other pathogenic *M. tuberculosis* complex species.

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