Direct Identification of Mycobacterium haemophilum in Skin Lesions of Immunocompromised Patients by PCR-Restriction Endonuclease Analysis

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PCR-restriction endonuclease analysis (PRA) was used for direct identification of Mycobacterium haemophilum in clinical specimens from immunocompromised patients. PRA correctly identified M. haemophilum in four smear-positive specimens. Direct identification by PRA takes 2 to 3 working days compared to the 3 to 5 weeks required for culture isolation and identification by conventional methods.

In recent years, Mycobacterium haemophilum has emerged as an important human pathogen (6, 10, 12), causing mainly opportunistic infections in severely immunocompromised patients with AIDS and those receiving immunosuppressive therapy after transplantation (1, 4, 7, 8, 12, 16). M. haemophilum has also been isolated from localized lesions in immunocompetent pediatric patients with cervical lymphadenopathy (3, 9). Superficial lesions such as cutaneous lesions and multiple skin nodules are not uncommon clinical presentations of M. haemophilum infection (6, 10, 12). Though necessary, culture isolation and identification of mycobacteria by conventional methods, especially for M. haemophilum, are time-consuming and laborious, usually taking 3 to 5 weeks (5). PCR-restriction endonuclease analysis (PRA) of an amplified 439-bp segment of the hsp65 gene encoding the 65-kDa heat shock protein has been successfully used for the rapid identification of mycobacterial isolates to the species level and has gained wide acceptance (11, 13, 14). This study is aimed at applying PRA as a means of rapid identification of M. haemophilum directly from acid-fast bacillus (AFB) smear-positive skin lesion specimens from immunocompromised patients.

Organisms. We used four superficial lesion specimens from three immunocompromised patients with suspected nontuberculous mycobacterial infections (two skin biopsy specimens from erythematous nodules, one skin abscess specimen from a patient’s left foot, and one pus drainage specimen from a patient with skin burrsitis). Reference strains M. haemophilum ATCC 29548, M. intracellulare ATCC 13950, M. tuberculosis ATCC 27294, and M. kansasii ATCC 35775 were utilized as controls for PRA.

Specimen processing. Specimens (1 g of skin biopsy specimen and 0.3 to 0.6 ml of pus and abscess drainage specimens) were digested and decontaminated using MycoPrep (Becton Dickinson). The derived sediment was used for smears and cultures. An aliquot of the original specimen was kept at −70°C for molecular analysis.

AFB microscopy. Auramine O fluorescent stain was used, and positive smears were counterstained with Ziehl-Neelsen stain to confirm the presence of AFB (5).

Mycobacterial cultures. Each specimen was inoculated into two sets of BACTEC 460 12B vials (Becton Dickinson) and Löwenstein-Jensen (LJ) medium slants (BBL, Becton Dickinson), and each set was incubated at either 30 or 37°C. In addition, one blood agar plate was inoculated and incubated at 30°C. Broth and solid medium cultures were held for 6 and 8 weeks, respectively, and examined periodically for growth. The culture isolates were identified using DNA probes (AccuProbe; Gen-Probe, San Diego, Calif.) and conventional biochemical tests (5).

PCR amplification. A method modified from the one originally described (14) was used for direct identification by PRA. From smear-positive specimens, bacteria were harvested by centrifugation at 6,000 × g for 5 min. DNA extracts were prepared using the QIamp DNA Mini kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer’s instructions. Ten microliters of purified target DNA was added to 60 μl of PCR SuperMix (Invitrogen Life Technologies, San Diego, Calif.) and 2 μl of each of primers TB11 (5′-ACCAACGAGTTGGTGTCACCAT) and TB12 (5′-CTTTGACACCGCATTCCCT). The mixture was amplified at 94, 55, and 72°C for 1 min each for 38 cycles and then held for a 10-min extension period at 72°C. Positive and negative amplification from control strains was included in each run. For culture confirmation, bacterial DNA from positive-culture isolates was similarly purified and amplified for comparison of PRA patterns.

Restriction endonuclease analysis. Amplified DNA was restricted using BstEII and HaeIII (New England Biolabs, Beverly, Mass.) as described previously (13, 14). Restriction fragments were electrophoresed using 3% Metaphor agarose (4-bp resolution; BioWhittaker Molecular Application, Rockland, Maine) and a Mini-Sub-Cell electrophoresis system (Scie-Plas, Warwickshire, United Kingdom) at 100 V for 1.5 to 2.0 h. PRA band sizes were estimated visually by comparison with the following molecular size markers: a 50-bp ladder (MBI Fermentas), a 100-bp ladder (BioWhittaker Molecular Application), and the PRA bands corresponding to control strains included in each run. Visual PRA identifications were made prior to and independent of culture isolation and identification.

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Sequencing of hsp65 gene PCR product. The amplified products from three clinical specimens were purified, and sequencing was performed with an ABI Prism 3100 DNA sequencer (Applied Biosystems); the primers TB11 and TB12 were used for cycle sequencing as described previously (2). The sequences were analyzed using the GenBank database (BLASTN 2.2.8 program).

The principal characteristics of the three patients and identification results are summarized in Table 1. All four specimens were AFB smear-positive by fluorochrome and Ziehl-Neelsen staining, with AFB score ranges (+ and ++++) as shown in Table 1. Specimens MC17735 and MC31558 were culture positive after 2 weeks, with the BACTEC 460 12B vials and blood agar plates showing optimum growth at 30°C. No visible growth was observed on LJ medium slants. Probe tests for the M. tuberculosis complex were negative for these two specimens, as were most biochemical tests. Tests for growth with X factor and pyrazinamidase were positive, hence confirming the identity of the isolates as M. haemophilum. Specimens MC29362, MC17735, and MC31558 were identified as M. haemophilum by matching each specimen’s PRA band pattern with that of reference strain M. haemophilum ATCC 29548.

![Base pairs](image)

FIG. 1. PRA patterns for reference strains and superficial lesion clinical specimens. Lanes: 1, 100-bp markers; 2, M. tuberculosis ATCC 27294; 3, M. intracellulare ATCC 13950; 4, M. kansasii ATCC 35775; 5, M. haemophilum ATCC 29548; 6, MC29362; 7, MC17735; 8, MC31558 (lanes 2 to 8 correspond to BstEII-digested amplicons); 9, 50-bp markers; 10, MC31558; 11, MC17735; 12, MC29362; 13, M. haemophilum ATCC 29548; 14, M. kansasii ATCC 35775; 15, M. intracellulare ATCC 13950; and 16, M. tuberculosis ATCC 27294 (lanes 10 to 16 correspond to HaeIII-digested amplicons). Isolates from clinical specimens MC29362, MC17735, and MC31558 were identified as M. haemophilum by matching each specimen’s PRA band pattern with that of reference strain M. haemophilum ATCC 29548.

Sequencing of hsp65 gene PCR product. The amplified products from three clinical specimens were purified, and sequencing was performed with an ABI Prism 3100 DNA sequencer (Applied Biosystems); the primers TB11 and TB12 were used for cycle sequencing as described previously (2). The sequences were analyzed using the GenBank database (BLASTN 2.2.8 program).

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patterns from the patients' specimens with patterns produced by the control strains and molecular size markers was easily achieved with simple electrophoresis and gel visualization apparatuses, making this method of direct identification and detection of mycobacteria quick and cost-effective compared to other methods such as gene sequencing (15, 17). Our preliminary study shows that PRA is a very useful tool for direct identification of M. haemophilum in AFB smear-positive skin lesion specimens from immunocompromised patients. A further study is under way to determine the cost benefits of applying this method to the direct identification of other mycobacteria in patient specimens in the clinical laboratory.

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REFERENCES