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Phenotypic and Molecular Characterization of Three Clinical Isolates of *Mycobacterium interjectum*

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Introduction of molecular biology-based technology into an Australian mycobacterial reference laboratory has resulted in the identification of three isolates of *Mycobacterium interjectum* in the past 12 months. Conventional phenotypic methods failed to identify the species of these isolates, and high-performance liquid chromatography found that only one of the three isolates had a mycolic acid pattern similar to that of the type strain. In contrast, all three isolates were rapidly identified as *M. interjectum* by 16S rRNA gene sequence analysis. Two isolates were recovered from the lymph nodes of children with cervical lymphadenitis, confirming the pathogenicity of this organism. However, the third isolate was obtained from the sputum of an elderly male with chronic lung disease without evidence of clinical or radiological progression, suggesting that isolation of *M. interjectum* should not imply disease. With the increasing use of molecular biology-based technology in mycobacterial laboratories, *M. interjectum* may be recognized more frequently as a pathogen or commensal organism.

Clinically significant disease caused by mycobacteria other than *Mycobacterium tuberculosis* has been increasingly recognized in recent years, partly due to improvements in laboratory isolation methods and the introduction of molecular biology-based methods for species identification (4). One recently described species, *M. interjectum*, has been reported as the causative agent of lymphadenitis in a child (12) and has been associated with a case of chronic destructive lung disease (3). The clinical, radiological, and laboratory findings strongly supported a pathogenic role for the organism in these two patients (18). The present report describes the use of conventional identification protocols, high-performance liquid chromatography (HPLC) and 16S rRNA gene sequence analysis, to identify *M. interjectum* isolates from another three patients.

CASE REPORTS

Patient 1. A healthy 2-year-old girl presented with a 3-week history of a swelling in the left neck which had been increasing in size. Examination revealed a 2-cm enlarged lymph node in the anterior triangle of the left neck. The node was nontender and had no overlying erythema. At operation, a markedly abnormal lymph node was found to be densely adherent to surrounding connective tissue, the contents were enucleated, and the remaining cavity was curetted. A *Mycobacterium* species was cultured from the enucleated tissue. The wound healed satisfactorily, and there was no recurrence after a 7-month period of clinical review.

Patient 2. A well 3-year-old girl presented with a 3-month history of left-sided cervical swelling which had been slowly increasing in size. Examination revealed a nontender, fluctuant, 3-cm mass in the anterior triangle of the left neck with no associated erythema. In addition, a mildly enlarged jugulodigastric node in the right neck was palpated. At operation, thick, purulent material was drained, the cavity was curetted thoroughly, and a sample was sent for culture. Histological examination of the cavity scrapings revealed florid necrotizing granulomatous inflammation, but no acid-fast bacilli were seen. Culture isolated a *Mycobacterium* species. Satisfactory wound healing was noted at outpatient review, and the child remains well 6 months after surgery.

Patient 3. An elderly male emigrated from El Salvador to Australia in 1992. Preemigration evaluation had revealed changes on a chest X ray, and although pulmonary tuberculosis was not confirmed, he received a 6-month course of standard antituberculous therapy before being allowed to travel to Australia. Postarrival X rays have demonstrated widespread interstitial lung opacities that have remained stable for the past 4 years. As part of the routine follow-up, three sputum specimens were obtained for mycobacterial investigations; an acid-fast bacillus was isolated from one specimen.

MATERIALS AND METHODS

Cultures. Lymph node tissue was processed without decontamination, inoculated onto Lowenstein-Jensen (L-J) medium, and incubated at 30 and 35°C in air. The sputum specimens were decontaminated with 2% sodium hydroxide and *N*-acetylcysteine for 30 min and then neutralized to pH 7, concentrated by centrifugation (3,000 × *g* for 15 min), and inoculated into a single Middlebrook 7H12 medium vial (BACTEC 12B; Becton Dickinson, Towson, Md.). All three clinical isolates failed to hybridize with commercially available probes for the *M. tuberculosis* complex and the *M. avium* complex (Accuprobe; Gen-Probe, Inc., San Diego, Calif.). By using defined procedures (17), the physical and biochemical properties of the clinical isolates and of the reference *M. interjectum* strain, DSM 44064 (12), were determined on two occasions and in parallel. Drug susceptibility testing was not performed.

HPLC. The HPLC method described by Butler et al. (1) was used to extract mycolic acids from colonies grown on L-J medium. An ODS2 reverse-phase C_{18} column (4.0 mm by 5.0 cm with 3-μm particles; Scientific Glass Engineering, Melbourne, Australia) was used in an ICI Scientific Instruments modular HPLC system comprising three LC1500 pumps controlled by a model 50 programmer, an AS2000 autosampler, a TC19000 column temperature controller, and a Knauer Variable Wavelength Spectrophotometer set at 260 nm. Data were collected via a Nelson Analytical 760 series interface and were analyzed by using PE Nelson Turbochrom 3 software, version 3.3 (4B11; Perkin-Elmer, Cupertino, Calif.). Peaks were identified on the basis of their retention times relative to those of the internal molecular weight standards (Ribi ImmunoChem Research, Hamilton, Mont.).

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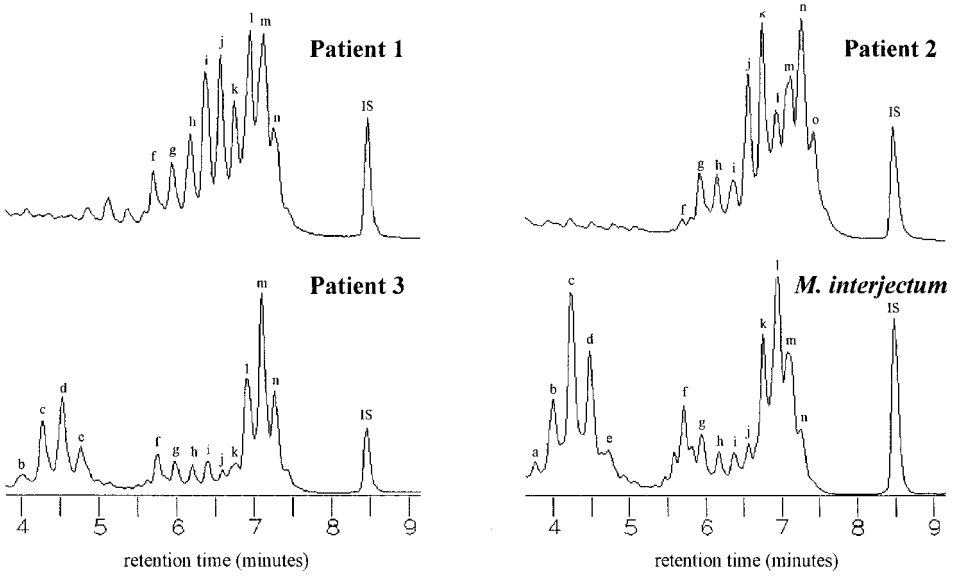

FIG. 1. HPLC profiles of clinical isolates and *M. interjectum* reference strain. IS, internal standard.

Molecular investigations. DNA was extracted from colonies grown on L-J medium by an established method (8). PCR primers were designed to amplify three overlapping products spanning the mycobacterial 16S rRNA gene: $G^{A}/_{G}G^{A}/_{G}$TACTCGAGTGGCGAAC and CCTACGAGCTCTTTACG (amplifying positions 93 to 586 as numbered in the *Escherichia coli* 16S rRNA sequence), CAGCAGCTGCGGTAATACG and CACATGCATACAGGCCACA AGG (positions 518 to 1050), AGAAGCTTACCTGGGTTTGAC and AAGGA $GG^{T}/_{G}$GATCCAGCCGC (positions 976 to 1542). For each primer pair, approximately 1 μg of mycobacterial DNA was contained in a reaction volume of 50 μl (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM [each] deoxynucleoside triphosphate, 0.5 μM [each] primer, and 1 U of *Taq* polymerase [Perkin-Elmer Cetus, Norwalk, Conn.]). A 5-min denaturation step at 94°C was followed by 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR products of the expected size were purified with QIAquick columns (Qiagen GmbH, Hilden, Germany) and were cycle sequenced by dye termination and with an automated system (Perkin-Elmer, Applied Biosystems Division, Foster City, Calif.).

Nucleotide sequence accession numbers. The GenBank accession numbers for the 16S rRNA genes of the *M. interjectum* isolates from patients 1, 2, and 3 are AF014935, AF014936, and AF014937, respectively.

RESULTS

Primary cultures positive for acid-fast bacilli were subcultured onto L-J-pyruvate- and L-J-glycerol-containing media and were incubated in air at 35°C. For all three isolates, small, nonpigmented, smooth-domed colonies were present on L-J-pyruvate medium after 4 weeks; only a faint growth haze was visible on L-J-glycerol medium. By week 6, the colonies had become pale yellow and the coloring had intensified with time. The isolates grew at temperatures from 25 to 37°C but not at 43°C and were weakly positive for arylsulfatase activity at 14 days. Negative results were obtained for nitrate reduction, 3-day tellurite reduction, and growth on L-J medium containing 5% sodium chloride. Growth was not affected by the presence or absence of 1.5% ferric ammonium citrate. Variable results were observed both between isolates and on repeated testing of the same isolate for Tween 80 hydrolysis and urease, semiquantitative catalase, and pyrazinamidase activities.

The HPLC profiles (Fig. 1) of the *M. interjectum* reference strain and the isolate from patient 3 were similar, with mycolic acid peaks eluting between 4.0 and 7.5 min with a biphasic pattern. However, the profiles of the isolates from patients 1 and 2 were significantly different, having a single cluster of peaks eluting between 5.5 and 7.5 min.

The initial sequence analyses were performed with PCR products amplified from the 5′ end of the mycobacterial 16S rRNA gene (i.e., positions 93 to 586), which contains hypervariable regions A and B (7, 9–11). The sequence of the 422-bp product amplified from the isolate from patient 3 was identical to the published *M. interjectum* sequence, and the products from the other two isolates differed by only 1 base substitution (Fig. 2). In view of the discordant phenotypic and HPLC results, the 16S rRNA studies were extended. A total of 1,351 bases were analyzed for each of the clinical isolates and the type strain. A high degree of homology (i.e., 99.6 to 99.9%) was demonstrated between these four organisms; the most diver-

	245	1114
M. tuberculosis[a]	AGC TTG TTG /	TTG TCT CAT GTT GCC
M. interjectum[a]	... .A. ...	..A .T. ... ...
Patient 1	... ...	... ...
Patient 2	... ...	... ...
Patient 3	... .A. ...	... ...

	1129	1262
M. tuberculosis[a]	AGC ACG TAA TGG /	GCC GCG AGG
M. interjectum[a]	... GG. C	... ..A ...
Patient 1	... G..	... ..A ...
Patient 2	... GG. C	... .TA ...
Patient 3	... GG. C	... ..A ...

FIG. 2. Alignment of variable regions within 16S rRNA sequences. [a], The sequences of the strains from patients were compared with published sequences for *M. tuberculosis* (EMBL accession no. X52917) and *M. interjectum* (EMBL accession no. X70961). Only nucleotides differing between *M. interjectum* and the patient isolates are presented. Dots indicate nucleotide identity. The relative *E. coli* 16S rRNA positions are indicated above the alignments.

gent pair, the type strain and the isolate from patient 1, differed by only 5 base substitutions.

DISCUSSION

With the increasing recognition of the importance of nontuberculous mycobacteria, diagnostic laboratories must provide prompt and accurate mycobacterial identifications so that clinicians can determine the significance of clinical isolates and select the most appropriate therapy. The methods for the identification of nontuberculous mycobacteria have been compared and reviewed recently (9, 13). Conventional identification methods based on phenotypic and biochemical characteristics have prolonged turnaround times of 3 to 6 weeks, require a skilled technician, may produce ambiguous results, and are essentially limited to identifying common species whose properties have been fully documented. Nucleic acid probes are available commercially for only a limited number of mycobacterial species. HPLC has been used in reference laboratories to identify mycobacteria (5, 14). However, the equipment is expensive, growth conditions and other variables in the methodology must be standardized, and a database of mycolic acid patterns from known species must be accumulated. In contrast, 16S rRNA gene analysis by amplification and direct sequencing is rapid, reliable, and increasingly available (7, 13); the gene sequence obtained is unaffected by growth conditions or other variables and can be easily compared with the GenBank or EMBL sequence entries for reference mycobacterial species.

The advantages and disadvantages of these mycobacterial identification techniques were highlighted while identifying the three new clinical isolates of *M. interjectum*. Definitive species identification was not possible by conventional phenotypic methods, despite repeated testing. These isolates would have been reported as slowly growing pigmented mycobacteria, with the differential diagnoses including *M. interjectum*, *M. gordonae*, and *M. scrofulaceum*. The original description of *M. interjectum* listed a negative result for Tween 80 hydrolysis and positive reactions for urease, pyrazinamidase, and heat-stable catalase (12). The three isolates described in this paper produced inconsistent results by these tests, precluding a definitive phenotypic identification. Interestingly, later descriptions of *M. interjectum* isolates have also listed variable reactions for these tests, particularly the urease reaction (3, 16, 19).

The HPLC and molecular biology-based studies also produced discordant results. The *M. interjectum* reference strain and the isolate from patient 3 had similar HPLC profiles but had mycolic acid patterns that differed from the patterns shared by the isolates from patients 1 and 2. HPLC profiles are reportedly species specific (1, 5, 14). The HPLC results therefore suggested that the isolate from patient 3 belonged to the *M. interjectum* species, but the other two clinical isolates did not. However, the sequences of all three patient isolates were 99.8 to 100% homologous to the published *M. interjectum* sequence on the basis of the initial analysis of a 422-bp segment from the 5′ end of the mycobacterial 16S rRNA gene. This segment contains B signature sequences in hypervariable regions A and that accurately differentiate mycobacterial species, with only a few exceptions (7, 9–11). The isolates from patients 1 and 2 therefore have the 16S rRNA signature sequences typical of *M. interjectum* but have atypical HPLC profiles.

Two explanations could account for the discrepancy between the HPLC and molecular biology-based assay findings. The *M. interjectum* species may contain variants with different mycolic acid patterns, as occurs with *M. gordonae* (2). Alternatively, the isolates from patients 1 and 2 could belong to a different mycobacterial species that happens to have the same signature sequences as *M. interjectum*. For instance, *M. ulcerans* and *M. marinum* have identical signature sequences, but extended 16S rRNA gene analysis will detect a single base substitution that differentiates these species (6). To clarify the situation, our molecular biology-based studies were extended to include 87.0% (i.e., 1,351 bases) of the 16S rRNA gene sequence of each clinical isolate and the type strain. This additional sequencing found 99.6 to 99.9% homology between the three clinical isolates and the type strain.

Two other recent reports describe mycobacterial isolates that have the same genotype as *M. interjectum* but atypical biochemical reactions and HPLC profiles. Tortoli et al. (16) isolated such an organism from the sputum of an immunocompromised AIDS patient. The other report (19) came from the International Working Group on Mycobacterial Taxonomy (IWGMT), which conducted extensive studies on 66 unusual, slowly growing mycobacteria and found three strains (belonging to branches H-2 and H-3 in the phylogenetic tree published previously [19]) that had molecular features in common with the *M. interjectum* type strain (i.e., homologous 16S rRNA sequences and high DNA-DNA hybridization levels) but that had atypical phenotypic characteristics and lipid patterns. In fact, the 1,351-bp 16S rRNA sequence of the isolate from patient 1 is identical to that of the single strain (i.e., IWGMT 90100) on branch H-2. Hence, on the basis of phenotypic and molecular biology-based assay results for the three isolates described in the present report and the IWGMT investigations, the most plausible explanation for these atypical strains with discrepant genotypic and phenotypic results is that the *M. interjectum* species contains variants with different HPLC profiles.

Only two clinically significant isolates of *M. interjectum* have been described previously. The original isolate was obtained from an 18-month-old boy with submandibular lymphadenitis (12). In the other report (3), *M. interjectum* was repeatedly isolated in sputum specimens from a woman with chronic destructive lung disease. A European group of investigators has described two other isolations of uncertain clinical significance (15, 16). The present report describes an additional two cases of pediatric lymphadenitis due to *M. interjectum* (or a closely related subspecies), confirming the pathogenicity of this organism. However, the isolation of *M. interjectum* in one of several sputum specimens from patient 3 who had no evidence of active disease suggests that this organism may also be a colonizer or a contaminant.

Twelve months ago, our mycobacterial reference laboratory introduced 16S rRNA sequence analysis for the identification of nontuberculous, non-*M. avium* complex mycobacteria. Introduction of this rapid and reliable technique resulted in the recognition of the three *M. interjectum* strains described in this paper. These identifications would not have been possible by conventional means. With more mycobacterial laboratories adopting these molecular biology-based techniques, *M. interjectum* (and related subspecies) may be identified with increasing frequency as pathogens or commensal organisms.

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