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Identification of *Mycobacterium neoaurum* Isolated from a Neutropenic Patient with Catheter-Related Bacteremia by 16S rRNA Sequencing

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A rapidly growing pigmented mycobacterial strain with an ambiguous biochemical profile was isolated from the blood culture taken through the Hickman catheter of a 9-year-old girl with acute lymphoblastic leukemia. Whole-cell fatty acid analysis showed that the best match profile was that of *Mycobacterium aurum*, but the similarity index was only 0.217, meaning that there were no good matches between the isolate and the organisms in the database of the Microbial Identification System. The 16S rRNA gene of the mycobacterial strain was amplified, agarose gel purified, and sequenced. There were 44 base differences between the gene sequence of the isolate and that of *M. aurum* but only one base difference between the sequence of the isolate and that of *Mycobacterium neoaurum*, showing that the isolate was indeed a strain of *M. neoaurum* by using this "gold standard." This represents the first case of *M. neoaurum* infection documented by 16S rRNA sequencing.

CASE REPORT

During the third course of intensification chemotherapy, a 9-year-old girl with acute lymphoblastic leukemia developed a fever of 38°C. Physical examination revealed no lymphadenopathy, hepatomegaly, splenomegaly, or other localizing signs. There were no signs of exit site infection of the Hickman catheter. Counts were as follows (per liter unless other units are given): hemoglobin, 8.7 g/dl; white blood cell, 1.9×10^9; neutrophil, 1.1×10^9; lymphocyte, 0.4×10^9; monocyte, 0.4×10^9; and platelet, 120×10^9. The hepatic enzymes, serum urea and creatinine levels, and serum albumin and globulin levels were within normal limits. Blood cultures were performed through the two lumens of the Hickman catheter, respectively, and were incubated using the BACTEC 9240 blood culture system (Becton Dickinson, Sparks, Md.). Intravenous ceftazidime and amikacin were given as empirical treatment for neutropenic fever.

On day 3 after admission, the blood culture taken from one of the lumens of the Hickman catheter turned positive with acid-fast bacilli. Pigmented colonies were observed on Lowenstein-Jensen medium after 3 days of incubation at 37°C. Conventional biochemical tests performed on the isolate failed to reveal a pattern identical to any known rapidly growing pigmented *Mycobacterium* species. It grew at 25, 31, and 37°C but not at 40 and 45°C. It tolerated 5% NaCl, reduced nitrate, produced urease, and was positive for iron uptake but was negative for catalase, Tween hydrolysis, and arylsulfatase. Sequencing of the 16S rRNA gene was therefore performed for the identification of the isolate to species level. Moreover, the isolate was sent to a local mycobacteriology reference laboratory for analysis of whole-cell fatty acid pattern by gas chromatography. An antimicrobial susceptibility test using the disk diffusion method with the standard disks on Mueller-Hinton agar showed zones of inhibition of ≥30 mm for ampicillin, amoxicillin-clavulanic acid, imipenem, meropenem, ofloxacin, ciprofloxacin, amikacin, tetracycline, doxycycline, and co-trimoxazole (1).

Although the neutropenic fever responded to ceftazidime and amikacin, subsequent blood cultures taken through the same lumen of the Hickman catheter on days 3, 6, and 13 were persistently positive for the same rapidly growing *Mycobacterium*, while all blood cultures performed through the other lumen were negative. Catheter-related bacteremia was diagnosed, and the Hickman catheter was removed. Blood culture performed 1 day after removal of the catheter was negative. Ceftazidime and amikacin were continued for a total of 3 weeks during neutropenia. The patient was put on maintenance chemotherapy and remained asymptomatic at the time of writing, 16 months from the time of bacteremia.

Microbiology. Whole-cell fatty acid analysis was performed using a Hewlett-Packard (Avondale, Pa.) 5890A gas chromatograph equipped with the Microbial Identification System (MIDI Inc., Newark, Del.). The fatty acids of the isolate were $C_{12:0}$ (0.29%), $C_{14:0}$ (5.9%), $C_{15:0}$ (0.43%), $C_{16:1}$ *cis*9 (4.24%), $C_{16:1}$ *cis*7 (0.82%), $C_{16:1}$ *cis*6 (2.97%), $C_{16:0}$ (25.48%), $C_{17:1}$ *cis*7 (12.88%), $C_{17:0}$ (0.4%), $C_{18:2}$ (0.29%), $C_{18:1}$ *cis*9 (34.49%), $C_{18:1}$ *cis*7 (0.79%), $C_{18:0}$ (4.62%), $C_{20:0}$ alcohol (3.18%), and $C_{20:0}$ (3.23%). The findings showed that the best match was *Mycobacterium aurum*, but the similarity index was only 0.217, meaning that there were no good matches between the isolate and the organisms in the database of the Microbial Identification System.

PCR amplification and DNA sequencing of the 16S rRNA gene was performed according to a published protocol (21). 5′-TGGCGAACGGGTGAGTAA-3′ (LPW81) and 5′-AGGCCCGGGAACGTATTCAC-3′ (LPW58) were used as the PCR primers, and 5′-TGGCGAACGGGTGAGTAA-3′ (LPW81), 5′-AGGCCCGGGAACGTATTCAC-3′ (LPW58), 5′-TTACTGGGCGTAAAGAGC-3′ (LPW99), 5′-TAATCCACATGCTCCGCC-3′ (LPW100), 5′-GCTCTTTACGCCCAGTA

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TABLE 1. Characteristics of our patient and the other three patients with *M. neoaurum* catheter-related bacteremia reported in the literature

Characteristic	Characteristics of patient no.:			
	1	2	3	4
Source	Davison et al. (4)	Holland et al. (8)	George et al. (5)	Woo et al. (in press)
Country	Australia	Australia	United States	Hong Kong
Sex[f]	F	M	M	F
Age (yr)	53	17	46	9
Underlying disease	Metastasized ovarian cancer	ALL[a]	Primary pulmonary hypertension	ALL
Reason for long-term central catheter insertion	Total parenteral nutrition	BMT[b]	Prostacycline infusion	Chemotherapy
Total WCC[c]/liter at bacteremia	NA[d]	0.1×10^9	4.7×10^9	1.9×10^9
ANC[e]/liter at bacteremia	NA	0.0	Normal	1.1×10^9
Antibiotics	Cefoxitin, gentamicin	Ticarcillin-clavulanate, tobramycin	None	Ceftazidime, amikacin
Removal of Hickman catheter	No	Yes	Yes	Yes
Response to treatment	Yes	Yes	Yes	Yes

[a] ALL, acute lymphoblastic leukemia.
[b] BMT, bone marrow transplantation.
[c] WCC, white blood cell count.
[d] NA, not available.
[e] ANC, absolute neutrophil count.
[f] M, male; F, female.

A-3′ (LPW102), and 5′-GGCGGAGCATGTGGATTA-3′ (LPW103) were used as the sequencing primers (Gibco BRL). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank by multiple sequence alignment using the CLUSTAL W program (18).

PCR of the 16S rRNA gene of the rapidly growing pigmented *Mycobacterium* showed a band at 1,273 bp. There were 44 base differences between the gene sequences of the isolate and *M. aurum* (GenBank accession no. X55595) but only one base difference between the isolate and *M. neoaurum* (GenBank accession no. M29564). Therefore the isolate was indeed a strain of *M. neoaurum* according to this "gold standard."

Discussion. Identification of rapidly growing pigmented mycobacteria traditionally relies on isolation of the mycobacterium and subsequent identification by phenotypic conventional biochemical tests or whole-cell fatty acid analysis. However, these two systems are associated with a lot of drawbacks. The turnaround time for identification is long when conventional tests are used, as some biochemical reactions may take up to 28 days to complete. Furthermore, some isolates exhibit ambiguous biochemical profiles and hence are unidentifiable. As for whole-cell fatty acid analysis using gas chromatography, special equipment and expertise are required and are generally not available in most routine clinical microbiology laboratories. Although commercially available molecular-based methods such as PCR (22), ligase chain reaction (9), and hybridization (12) have revolutionized the rapid identification of some mycobacteria, such as *M. tuberculosis* and *M. avium*, no commercially available PCR or ligase chain reaction is available for the identification of rapidly growing pigmented mycobacteria, as this group of mycobacteria is rarely encountered in clinical specimens.

Small subunit rRNA sequencing, particularly 16S rRNA sequencing in bacteria, has led to advances on multiple fronts in microbiology. Firstly, the construction of a universal phylogenetic tree classifies organisms into three domains of life: *Bacteria*, *Archaea*, and *Eucarya* (10, 11, 20). Secondly, it revolutionizes the classification of microorganisms and makes the classification of noncultivable microorganisms possible (13, 14). Thirdly, it helps to elucidate the phylogenetic relationship of novel bacterial species to known ones. New species of mycobacteria such as *M. genavense* (2), *M. heidelbergense* (6), *M. hassiacum* (15), *M. novocastrense* (16), *M. conspicuum* (17), and *M. tusciae* (19) have been discovered using 16S rRNA sequencing. Moreover, mycobacteria such as *M. celatum*, which was not known to cause infections in humans, have been identified in clinical specimens using this technique (3). In this study, we report the first case of *M. neoaurum* infection in which the isolate with an ambiguous biochemical profile was identified by 16S rRNA sequencing.

The present report represents the fourth case of *M. neoaurum* infection in the English literature (Table 1). All four patients who had *M. neoaurum* infection suffered from Hickman catheter-related bacteremia (4, 5, 8). The male/female ratio is 1:1, with a median age of 26.5 (range, 9 to 53). Three patients had underlying malignancies, and two had neutropenia at the time of bacteremia. Removal of the Hickman catheter was required for three patients, whereas antibiotic treatment was sufficient to cure the infection in one. All four patients responded to treatment, with no relapse of the bacteremia. Antibiotic susceptibility testing was performed on three of the four isolates (Table 2). Antimicrobial agents with zone inhibition of $\geq$30 mm included the carbapenems, the quinolones, amikacin, and the tetracyclines. The ideal choice for the treatment of *M. neoaurum* infections in the future probably should include a combination of two of the four agents (e.g., carbapenem plus amikacin or carbapenem plus quinolone), and Hickman catheter removal would be necessary if there is persistent bacteremia after antibiotic therapy.

16S rRNA sequencing will continue to be the gold standard for identification of *Mycobacterium* spp., and the automation of such a technique may put it into routine use in clinical microbiology laboratories, replacing the traditional phenotypic tests and whole-cell fatty acid analysis. Modern technologies have made it possible to construct a high density of oligonucleotide arrays on a chip with oligonucleotides representing the 16S rRNA sequence of various mycobacteria, including those rarely encountered in clinical specimens. Such a design will facilitate automation of the annealing and detection of the PCR products of 16S rRNA amplification, and hence routine identification of most clinical isolates will be possible. The use of 16S rRNA sequencing has several advantages. Firstly, the

TABLE 2. Zones of inhibition of *M. neoaurum* isolated from patients with catheter-related bacteremia

Antibiotic	Zone diameter (mm) for isolate from patient:		
	1	2	4
Ampicillin			30
Amoxicillin	18		
Amoxicillin-clavulanate			45
Cefazolin			16
Cefuroxime			15
Cefoxitin	≥30	≥30	23
Enoxacin	≥30		
Cefotaxime	6		
Ceftazidime			6
Ticarcillin-clavulanate		≥30	
Imipenem	≥30	≥30	48
Meropenem			41
Erythromycin		≥30	16
Clarithromycin		≥30	24
Roxithromycin		≥30	
Azithromycin		≥30	
Vancomycin	≥30		
Ofloxacin			52
Ciprofloxacin		≥30	60
Amikacin	≥30	≥30	45
Tobramycin		25	23
Gentamicin	≥30		
Kanamycin	≥30		
Tetracycline	≥30	≥30	55
Doxycycline	≥30		65
Minocycline	≥30		
Co-trimoxazole	≥30	<20	35

turnaround time would be markedly shortened. Since amplification of the 16S rRNA gene takes only 4 to 6 h and the annealing and detection of PCR product takes only another few hours, theoretically the identification can be completed within 1 day. Secondly, oligonucleotides representing all *Mycobacterium* species, including those rarely encountered clinically, can be included in the array, making it easy to identify the rare species. Thirdly, the problem of unidentifiable strains will be overcome and there would be minimal misidentification, as the identification of a clinical strain is clearly defined by the number of base differences between it and the existing species. In the recently published study where only 6 of 11 rapidly growing mycobacteria were correctly identified by conventional tests and lipid profiles compared to 16S rRNA sequencing, all two *M. neoaurum* strains were either misidentified as *M. vaccae* or could not be identified (7). Lastly, unlike whole-cell fatty acid analysis, such a technique will be applicable to not only mycobacteria but also other bacteria. Therefore, it will reduce not only manpower but also capital costs and costs of consumables in the long run. Although the cost-effectiveness of using 16S rRNA sequencing for routine identification of *Mycobacterium* species remains to be evaluated, the present example shows the usefulness of 16S rRNA sequencing for the identification of a mycobacterial strain with an ambiguous biochemical and fatty acid profile.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *M. neoaurum* was submitted to GenBank and given accession no. AF268445.

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