JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2008, p. 1178–1184 Vol. 46, No. 4
0095-1137/08/$08.00+0 doi:10.1128/JCM.02011-07

Nocardia wallacei sp. nov. and *Nocardia blacklockiae* sp. nov., Human Pathogens and Members of the "*Nocardia transvalensis* Complex"▽

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Received 12 October 2007/Returned for modification 21 December 2007/Accepted 28 January 2008

Nocardia **isolates that share the property of in vitro amikacin resistance are grouped together by some authors in the *Nocardia transvalensis* complex. Our examination of 13 isolates that are amikacin resistant has revealed the existence of three distinct species. Sequence analysis of the 16S rRNA, 65-kDa heat shock protein, and *secA1* genes, coupled with DNA-DNA hybridization, indicated that "*N. asteroides* drug pattern IV," "*N. transvalensis* new taxon 1," and *N. transvalensis* sensu stricto should each be considered a distinct species. The phenotypic and molecular characteristics of the proposed new species *Nocardia wallacei* (*N. asteroides* drug pattern IV) and *N. blacklockiae* (*N. transvalensis* new taxon 1) are presented and compared with those of *N. transvalensis* sensu stricto. The relative genetic diversity of isolates best placed with the species *N. blacklockiae* is also discussed. Case studies demonstrating the pathogenicity of *N. wallacei* and *N. blacklockiae* are presented. The type strain of *N. wallacei* is ATCC 49873 (DSM 45136), and that of *N. blacklockiae* is ATCC 700035 (DSM 45135).**

Nocardia transvalensis was first described by Pijper and Pullinger in 1927 as the causative agent of mycetoma of the foot in a South African patient (17); this isolate was later characterized biochemically by Gordon et al. (11). *N. transvalensis* has since been found to be an uncommon but significant pathogen capable of causing disseminated infections, particularly in immunocompromised patients. In 1992, McNeil et al. reported on the recovery of *N. transvalensis* from 16 patients from Australia, Canada, Nigeria, Spain, and the United States, 10 of whom had clinical and/or histopathologic evidence of infection (16). Of these patients with proven pulmonary or disseminated infection, six had definable underlying immunosuppressive conditions. While susceptibility to specific antimicrobials varied among the isolates, it was apparent that these *N. transvalensis* isolates showed high levels of antibiotic resistance, especially to the aminoglycosides. In addition, the results of biochemical testing showed considerable variability among the isolates, suggesting the presence of several biotypes within the *N. transvalensis* group.

In 1988, Wallace et al. reported the presence of six patterns of antibiotic susceptibility among *Nocardia* isolates identified biochemically as *N. asteroides* (19). One of these patterns, drug pattern IV, showed a high level of resistance to aminoglycosides, including amikacin. Using amplification and subsequent restriction endonuclease analysis (REA) of a portion of the 65-kDa heat shock protein gene (HSP), Steingrube et al. were able to differentiate three restriction fragment length polymorphisms (RFLPs) in isolates determined to be amikacin resistant (18). Isolates identified phenotypically as *N. asteroides* drug pattern IV showed a single unique RFLP pattern, while isolates identified as *N. transvalensis* showed one of two different RFLP patterns.

In 1997, Wilson et al. (23) examined the biochemical characteristics, molecular differences (as determined by REA of a portion of the HSP gene), and cell wall composition of *Nocardia* isolates that were determined to be resistant to amikacin or that had been determined phenotypically to belong to *N. transvalensis*. On the basis of their results, isolates identified as *N. asteroides* drug pattern type IV were determined to be more closely related to *N. transvalensis* than to other members of the *N. asteroides* complex. Isolates of their *N. transvalensis* complex were assigned to one of four distinct groups: *N. transvalensis* sensu stricto, *N. asteroides* drug pattern IV, and *N. transvalensis* new taxons 1 and 2. Wilson et al. found that these four groups were best defined using REA of the HSP gene with six restriction endonucleases (23).

Here we describe our findings regarding the molecular, biochemical, and susceptibility characteristics of the reference strains of *N. asteroides* drug pattern type IV, and *N. transvalensis* new taxon 1, and clinical isolates assignable to two of these three taxonomic groups. In addition, we compare these findings to the molecular and biochemical characteristics of *N. transvalensis* sensu stricto. As no reference strain for *N. transvalensis* new taxon 2 is available, we did not pursue the study of this taxon. We also propose species designation for *N. asteroides* drug pattern IV (*N. wallacei*) and *N. transvalensis* new

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▽ Published ahead of print on 6 February 2008.

taxon 1 (*N. blacklockiae*). Additionally, we provide data confirming the clinical significance of these two proposed species.

CASE REPORTS

Case 1 (isolate 1, *N. wallacei*). The patient was a 57-year-old male who presented in 1979 with a 3-week history of chills, fever, cough, and left pleuritic pain. He had a history of carcinoma of the tongue that had been treated with radiotherapy and *Pneumocystis jirovecii* pneumonia that had been diagnosed by an open lung biopsy 2 years earlier. On admission, the patient was found to have a new extensive left lung infiltrate and a left pleural effusion. No results could be found for an acid-fast stain, but Gram stain results indicated sparse *Nocardia*-like organisms and many polymorphonuclear cells. Cultures of pleural fluid and sputum grew a *Nocardia* species, as determined by phenotypic methods. The organism was later identified as *N. asteroides* drug pattern IV by REA of the HSP gene. Susceptibility testing was performed by disk diffusion at a reference laboratory and indicated resistance to amikacin. Subsequent testing at the Centers for Disease Control and Prevention (CDC) confirmed the resistance of this isolate to multiple drugs, including amikacin. The patient became afebrile within 24 h of beginning trimethoprim-sulfamethoxazole treatment. Immunologic evaluation revealed defective macrophage function, but lymphocyte studies revealed no abnormalities. The patient became febrile again, with progression of his lung infiltrates, after 4 months of therapy. He developed an altered mental status and aseptic meningitis. Therapy with multiple additional agents including amikacin failed, and he died of his disease.

Case 2 (isolate 6, *N. blacklockiae*). A 37-year-old Australian aborigine with a history of chronic alcoholism presented with a 3-week history of fever, chest pain, and cough with purulent sputum production. A chest radiograph showed consolidation and volume loss of the left upper lobe. Microscopy of Gram-stained sputum showed gram-positive branching filamentous organisms, and testing with a modified acid-fast stain was positive. Cultures of sputum grew a *Nocardia* species that was identified as *N. transvalensis* by phenotypic characteristics. The organism was later identified as a member of the *N. transvalensis* complex by REA of the HSP gene. MICs were determined in a reference laboratory and indicated resistance to amikacin. The patient was initially treated with intravenous gentamicin and sulfadiazine; after 1 week the antibiotics were changed to oral sulfadimidine and trimethoprim-sulfamethoxazole. A chest radiograph after 14 days of therapy demonstrated persistent left upper lobe consolidation and new cavitation. The drug regimen was again modified to include sulfadimidine, amoxicillin, and amikacin. Clinical and radiographic improvements were evident after 3 weeks of antibiotic treatment; however, considerable residual fibrosis remained in the left upper lobe. This case was previously reported in a discussion of the antibiotic susceptibility of *N. transvalensis* and of the spectrum of clinical illness attributed to the organism (16).

MATERIALS AND METHODS

Organisms. The ATCC reference strains of *N. wallacei* (ATCC 49872 and ATCC 49873 [proposed as the type strain of *N. wallacei*], and ATCC 700035 [proposed as the type strain of *N. blacklockiae*]) and the type strain of *N. transvalensis* sensu stricto (ATCC 6865) were used in this study. Patient isolates were obtained from the Children's Hospital and Regional Medical Center, Seattle, WA (one isolate), from isolates referred for identification to ARUP Laboratories (three isolates), or from isolates referred for identification and/or susceptibility testing to the University of Texas Health Center at Tyler (five isolates).

N. wallacei isolate 1 (case study 1) and isolates 2 through 5 were all recovered from sputum samples; *N. blacklockiae* isolate 6 (case study 2) and isolates 7, 8, and 9 were recovered from sputum, a brain abscess, a corneal ulcer, and a bronchial wash specimen, respectively. Except for *N. blacklockiae* isolate 6 (case study 2), the clinical significance of the respiratory isolates has not been determined; however, the *N. blacklockiae* isolates from the brain abscess and the corneal ulcer are presumably clinically relevant.

Phenotypic identification. Colonies were examined for aerial hyphae by using a dissecting microscope; microscopic characteristics were determined by Gram stain and by modified Kinyoun acid-fast stain. Biochemical tests were performed at the Actinomycete Reference Laboratory of the CDC by the methods of Berd (1). Utilization of acetamide as the sole carbon and nitrogen sources was determined as described by Wallace et al. (20); utilization of citrate as the sole carbon source was determined as described by Yassin et al. (24). Arylsulfatase production tests were performed as described by Kent and Kubica (12). Esculin hydrolysis testing was performed on solid medium (Remel, Lenexa, KA), as described by Williams et al. (22).

Susceptibility testing. Susceptibility testing was performed in two laboratories; isolates considered to belong to the species *N. blacklockiae* were tested in duplicate in each laboratory; isolates considered to belong to the species *N. wallacei* were tested only once in each laboratory. In laboratory 1 (CDC), the organisms were grown at 35°C in 25 ml of Middlebrook 7H9 broth (Difco, Sparks, MD) with albumin-dextrose-catalase enrichment (Gibco, Carlsbad, CA) by using glass beads and continuous shaking (generally for 3 days) until good growth was achieved. A standardized suspension was prepared from the supernatant by using Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (4). In laboratory 2 (National Institutes of Health [NIH]), *Nocardia* colonies from a 3- to 5-day growth on sheep blood agar (Remel) were crushed in 0.5 ml of sterile water with a pellet pestle (Kimble Chase, Vineland, NJ). The larger organism clumps were allowed to settle; a standardized suspension was prepared from the supernatant by using CLSI guidelines (4). In each laboratory, 10 μl of a standardized inoculum was added to each well of a commercially prepared, frozen microdilution plate (PML Microbiologicals, Wilsonville, OR) containing antimicrobial agents diluted in 100 μl of cation-supplemented Mueller-Hinton broth per well. The plates were incubated at 35°C for 72 h in ambient air; endpoints were determined according to the CLSI guidelines (4). The interpretive breakpoints used for all drugs were those recommended by the CLSI.

Gene sequencing. DNA was extracted from all reference strains, type strains, and patient isolates as described previously (7). 16S rRNA gene sequences (1460 bp), HSP gene sequences (441 bp), and *secA1* gene sequences (520 bp) were determined as described previously (6, 7, 9). The sequences of all genes were assembled and aligned by using Lasergene SeqMan II and MegAlign software (DNAStar, Inc., Madison, WI). The deduced amino acid sequence of the SecA1 protein was determined with MegAlign software. To determine sequence similarity, all sequences were cut to the size of the shortest sequence. Percent similarity was determined by counting the number of base differences and relating the number of these differences to the sequence length. For determination of percent similarity, any ambiguous bases were counted as mismatches.

DNA-DNA hybridization. Purified DNA of the designated type strains of *N. wallacei*, *N. blacklockiae*, and *N. transvalensis* and from the patient isolates was prepared from lysed protoplasts, as described previously (8, 14). In two separate experiments, reference strains were labeled with [^{32}P]dCTP by using a nick translation system (Invitrogen, Carlsbad, CA). In experiment 1, labeled DNA from the type strain of *N. wallacei* (ATCC 49873) was hybridized with the unlabeled DNA from isolates 1 through 5 and with the unlabeled DNA from *N. transvalensis*. In experiment 2, labeled DNA from the type strain of *N. blacklockiae* (ATCC 700035) was hybridized with the unlabeled DNA from isolates 6 through 9 and with the unlabeled DNA from *N. transvalensis*. Hybridization was performed as described previously (3). All reactions were performed in duplicate at 70°C. The relative binding ratio (RBR) was calculated by the method of Brenner et al. (2) as follows: (percentage of heterologous DNA bound to hydroxyapatite/percentage of homologous DNA bound to hydroxyapatite) × 100. The percent divergence (*D*; calculated to the nearest 0.5%) was determined by assuming that each degree of heteroduplex instability compared to the melting temperature of the homologous duplex was caused by 1% unpaired bases (2). An

TABLE 1. Biochemical characteristics of the type strains of *N. transvalensis* sensu stricto, *N. wallacei*, and *N. blacklockiae*; the reference strain of *N. wallacei*; and the patient isolates of *N. wallacei* and *N. blacklockiae*

Test	Result for[a]:					
	N. transvalensis ATCC 6865^T	*N. wallacei* ATCC 49873^T	*N. wallacei* reference strain ATCC 49872	*N. wallacei* patient isolates 1 to 5 (n = 5)	*N. blacklockiae* ATCC 700035^T	*N. blacklockiae* patient isolates 6 to 9 (n = 4)
Acid from oxidation of:						
Adonitol	+	−	−	−	+	+
Arabinose	−	−	−	−	−	−
Cellobiose	−	−	−	−	−	−
Dulcitol	−	−	−	−	−	−
i-Erythritol	+	−	−	−	+	+
D-Fructose	+	−	−	−	+	+
D-Galactose	+	+	+	+	−	+
D-Glucose	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+
i-myo-Inositol	+	−	−	−	−	−
Lactose	−	−	−	−	−	−
Maltose	+	+	+	+	+	+
D-Mannitol	+	−	−	−	+	+
Mannose	−	−	−	−	+	V
Melibiose	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−
L-Rhamnose	−	−	−	−	−	−
Salicin	+	+	+	V	+	V
D-Sorbitol	+	−	−	−	+	+
Sucrose	−	W+	−	−	−	V
Trehalose	+	+	+	+	+	+
D-Xylose	−	−	−	−	−	−
Growth on heart infusion agar:						
At 25°C	3+	2+	2+	2 + to 3 +	3+	3+
At 35°C	3+	3+	3+	2 + to 3 +	3+	3+
At 45°C	−	1+	2+	1+ to 2 +	−	−
Growth in lysozyme	+	+	+	+	+	+
Arylsulfatase production at:						
3 days	−	−	−	−	−	−
14 days	−	−	−	−	−	−
Hydrolysis of:						
Adenine	−	−	−	−	−	−
Casein	−	−	−	−	−	−
Esculin	+	+	+	+	+	+
Hypoxanthine	+	+	+	+	+	+
Tyrosine	−	−	−	−	−	−
Urea	+	+	+	+	+	+
Xanthine	−	−	−	−	−	−
Utilization of acetamide as the sole carbon and nitrogen source	−	−	−	−	−	−
Utilization of citrate (1%) as a sole carbon source	+	+	+	+	+	+

[a] Abbreviations: +, positive; −, negative; W+, weakly positive; V, variable; 1+, scant growth; 2+, moderate growth; 3+, heavy growth.

RBR greater than 70% with less than 6% *D* is considered indicative of conspecificity (21).

G+C content. The G+C content of DNA for the type strains of *N. wallacei* and *N. blacklockiae* was determined spectrophotometrically by thermal denaturation, as described previously (15).

Nucleotide sequence and culture collection accession numbers. The 16S rRNA, HSP, and *secA1* gene sequences of the *N. wallacei* and *N blacklockiae* type strains and of patient isolates 1, 6, and 7 have been submitted to GenBank and assigned accession numbers EU099357 through EU099371, respectively.

The *N. wallacei* and *N. blacklockiae* strains have been deposited in culture collections under the indicated strain numbers: *N. wallacei*, ATCC 49873^T and DSM 45136^T; *N. blacklockiae*, ATCC 700035^T and DSM 45135^T.

RESULTS

Phenotypic identification. For the five patient isolates considered to belong to the species *N. wallacei*, the biochemical results were identical to those obtained for the type strain

TABLE 2. Susceptibility results for the type strain, the reference strain, and patient isolates of *N. wallacei*[a]

Antimicrobial agent	Result for *N. wallacei*:			
	ATCC 49873^T	Reference strain ATCC 49872	Patient isolate 1	Patient isolates 2–5
Amikacin	R	R	R	R
AMC	S/I[b]	S	S	S
Ceftriaxone	S	S	S/I[b]	S/I[b]
Ciprofloxacin	S	S	S	S/R[b]
Clarithromycin	R	R	R	R
Imipenem	I/R[b]	R	R	R
Linezolid[c]	S	S	S	S
Minocycline	I	I	I	S/I[b]
SMX	S/R[b]	S	S/R[b]	S/R[b]
SXT	S/R[b]	S/R[b]	S/R[b]	S/R[b]

[a] Based on a single susceptibility test of each strain performed in two laboratories. Breakpoints are based on those reported in CLSI document M24-A (4). Abbreviations: AMC, amoxicillin-clavulanic acid; SMX, sulfamethoxazole; SXT, trimethoprim-sulfamethoxazole; R, resistant; S, susceptible; I, intermediate.
[b] Results varied between laboratories.
[c] Based on the proposed breakpoint (4).

except for the production of acid from the oxidation of sucrose and of salicin (for which the patient isolates gave variable results) (Table 1).

For the four patient isolates considered to belong to the species *N. blacklockiae*, variable results were obtained for the production of acid from the oxidation of mannose, salicin, and sucrose; and all patient isolates produced acid from the oxidation of D-galactose. Otherwise, the biochemical results for the patient isolates were identical to those for the *N. blacklockiae* type strain.

Susceptibility testing. As was expected for the members of the *N. transvalensis* complex, all type and reference strains and all patient isolates showed in vitro resistance to amikacin (data not shown) (23). For the type strain of *N. wallacei*, the susceptibility testing results (obtained once for each isolate in each laboratory) varied between laboratories for amoxicillin-clavulanic acid, imipenem, sulfamethoxazole, and trimethoprim-sulfamethoxazole (Table 2).

The susceptibility testing results for the type strain and patient isolates of *N. blacklockiae* showed not only interlaboratory variation but also intralaboratory variation, with the exception of amikacin resistance and linezolid susceptibility (data not shown).

Gene and amino acid sequences. Comparison of a 1,388-bp region of the 16S rRNA gene sequences of the type strains of *N. blacklockiae*, *N transvalensis*, and *N. wallacei* showed between 98.0 and 98.9% similarity among the strains (Table 3). Comparison of a 394-bp region of the HSP genes of these three isolates showed between 94.4 and 97.7% sequence similarity. Comparison of a 464-bp region of the *secA1* genes of the three isolates showed between 95.9 and 97.0% sequence similarity; the deduced SecA1 protein amino acid sequences showed between 97.4 and 98.7% sequence similarity (Table 3).

TABLE 4. Comparison of the 16S rRNA, HSP, and *secA1* gene sequences and DNA-DNA hybridization results for five patient isolates and one ATCC reference strain of *N. wallacei* with those for *N. wallacei* ATCC 49873^T

Isolate	% Similarity to *N. wallacei* ATCC 49873^T			DNA-DNA hybridization with *N. wallacei* ATCC 49873^T (RBR/% *D*)
	16S rRNA gene sequence (1,353 bp)	HSP gene sequence (394 bp)	*secA*1 gene sequence (464 bp)	
ATCC 49872	99.8	100	100	86/1.0
1	>99.9	100	100	89/1.0
2	>99.9	100	100	100/2.0
3	100	100	100	100/1.5
4	>99.9	99.8	100	84/1.5
5	>99.9	100	100	100/1.5

The 16S rRNA gene sequences of five patient isolates (isolates 1 through 5; 1,353 bp) showed ≥99.9% sequence similarity to the 16S rRNA gene sequence of the *N. wallacei* type strain (ATCC 49873) (Table 4). The 16S rRNA gene sequence of *N. wallacei* reference strain ATCC 49872 showed 99.8% similarity to that of the *N. wallacei* type strain (Table 4); the sequence discrepancies noted between these two strains (3 bp) were due in part to the presence of two ambiguous bases in the 16S rRNA gene sequence of ATCC 49872. Patient isolates 1 through 5 showed ≥99.8% gene sequence similarity to the *N. wallacei* type strain for both the HSP gene (394 bp) and the *secA1* gene (464 bp) (Table 4). The deduced amino acid sequence of the SecA1 protein for all patient isolates and the *N. wallacei* reference strain (ATCC 49872) showed 100% similarity to the deduced amino acid sequence of the *N. wallacei* type strain (data not shown).

The 16S rRNA gene sequence of isolate 6 (case 2) showed 99.9% similarity to that of the type strain of *N. blacklockiae* (1407 bp) (Table 5); the only differences between the two sequences were two base insertions in the sequence of the type strain. Analysis of the HSP gene (394 bp) and the *secA1* gene (464 bp) sequences showed that the two gene sequences of isolate 6 were 99.8 and 99.1% similar to the two gene sequences of the *N. blacklockiae* type strain, respectively (Table 5). The amino acid sequence of the SecA1 protein was identical to that of the type strain (data not shown).

Isolates 7, 8, and 9 each showed 99.3% 16S rRNA gene

TABLE 3. Comparison of 16S rRNA, HSP, and *secA1* gene sequences and SecA1 amino acid sequences of type strains of *N. wallacei* (ATCC 49873), *N. blacklockiae* (ATCC 700035), and *N. transvalensis* sensu stricto (ATCC 6865)

Species	% Similarity							
	N. blacklockiae				*N. transvalensis* sensu stricto			
	16S rRNA gene (1,388 bp)	HSP gene (394 bp)	*secA1* gene (464 bp)	SecA1 amino acid (155 residues)	16S rRNA gene (1,388 bp)	HSP gene (394 bp)	*secA1* gene (464 bp)	SecA1 amino acid (155 residues)
N. wallacei	98.0	97.7	97.0	98.7	98.6	94.4	95.9	97.4
N. blacklockiae					98.9	95.9	96.6	97.4

TABLE 5. Comparison of the 16S rRNA, HSP, and *secA1* gene sequences and DNA-DNA hybridization results for four patient isolates with those for the type strain of *N. blacklockiae* ATCC 700035^T

Isolate	% Sequence similarity to *N. blacklockiae* ATCC 700035^T			DNA-DNA hybridization with *N. blacklockiae* ATCC 700035^T (RBR/% *D*)
	16S rRNA gene sequence (1,407 bp)	HSP gene sequence (394 bp)	*secA1* gene sequence (464 bp)	
6	99.9	99.8	99.1	93/0.0
7	99.3	99.8	98.7	85/0.5
8	99.3	99.5	98.5	93/0.0
9	99.3	99.8	98.7	98/1.0

TABLE 6. DNA relatedness among the type strains of *N. blacklockiae, N. transvalensis*, and *N. wallacei*

Source of labeled DNA	Source of unlabeled DNA	RBR (% *D*)
N. wallacei	*N. wallacei*	100
N. wallacei	*N. transvalensis*	44 (10.5)
N. wallacei	*N. blacklockiae*	53 (8.5)
N. blacklockiae	*N. blacklockiae*	100
N. blacklockiae	*N. wallacei*	45 (7.5)
N. blacklockiae	*N. transvalensis*	34 (8.5)

sequence similarity to the 16S rRNA gene sequence of the *N. blacklockiae* type strain, with 10 base differences each, and they were more similar to each other than to the *N. blacklockiae* type strain (99.4 to >99.9% similarity). In addition, isolates 8 and 9 contained one and two ambiguous bases, respectively, that could not be resolved by repeat testing. Analysis of the HSP and *secA1* gene sequences for isolates 7, 8, and 9 showed ≥99.5 and ≥98.5% sequence similarities to the sequences of these two genes of the *N. blacklockiae* type strain, respectively. For isolates 7, 8, and 9, the deduced amino acid sequences of the SecA1 protein were 100% similar to the deduced amino acid sequence of the SecA1 protein of the *N. blacklockiae* type strain (data not shown).

DNA-DNA hybridization. DNA-DNA hybridization studies performed with the type strains of *N. wallacei*, *N. blacklockiae*, and *N. transvalensis* showed the strains to be sufficiently different to warrant their inclusion in three separate species (Table 6).

DNA-DNA hybridization of patient isolates 1 through 5 with the *N. wallacei* type strain showed RBRs of ≥84 and *D* values of ≤2.0% (Table 4). DNA-DNA hybridization of isolates 6 through 9 with the *N. blacklockiae* type strain showed RBRs of ≥85 and *D* values of ≤1% (Table 5).

G+C content. The G+C content of the type strains of both *N. blacklockiae* (ATCC 700035) and *N. wallacei* (ATCC 49873) was 65.0 mol%. This value is consistent with the G+C content observed in the genus *Nocardia* (64 to 72 mol%) (13).

DISCUSSION

The results of the present study confirm the differences among the type and reference strains of three of the four groups of the *N. transvalensis* complex. The type strains of *N. wallacei*, *N. blacklockiae*, and *N. transvalensis* sensu stricto show biochemical differences similar to those noted by Wilson et al. (23) (Table 1). Sequence analysis of the 16S rRNA, HSP, and *secA1* genes showed considerable molecular differences among these type and reference strains (Table 3). In addition, the DNA-DNA hybridization results for the type strains of these species confirm the molecular distinctness of these three groups (Table 6).

In the United States, *N. wallacei* is the most commonly isolated member of the *N. transvalensis* complex (23). In the 1988 study of Wallace et al. (19), this organism represented 5% of the *Nocardia* isolates submitted for susceptibility testing to the University of Texas Health Center in Tyler. In the present study, isolates determined to belong to the species *N. wallacei* showed considerable homogeneity in their biochemical characteristics (Table 1) and, except for the sulfonamides, in their susceptibility testing results (Table 2). The 16S rRNA, HSP, and *secA1* gene sequences of the clinical isolates of *N. wallacei* were extremely similar to those of the type strain of the species, in addition to being molecularly similar to each other. DNA-DNA hybridization confirmed their conspecificity.

N. blacklockiae is similar to other members of the *N. transvalensis* complex in its resistance to amikacin; the results of molecular analysis presented here show that this species is distinct from the other members of the complex.

Three of the four isolates (patient isolates 7, 8, and 9) have been designated here as belonging to the species *N. blacklockiae*, despite the presence of some notable 16S rRNA gene sequence differences from the type strain. Although the HSP and *secA1* gene sequences and the DNA-DNA hybridization results all place these isolates within the species *N. blacklockiae*, the similarity of the 16S rRNA gene sequences of these isolates to the 16S rRNA gene sequence of the *N. blacklockiae* type strain is only 99.3%. This is a lower percentage of similarity than that seen for other isolates unequivocally belonging to the same *Nocardia* species, which are usually ≥99.9% similar. The 99.3% similarity of the 16S rRNA genes of isolates 7, 8, and 9 to the 16S rRNA gene sequence of the type strain of *N. blacklockiae* is also less than the 16S rRNA gene sequence similarity of some distinct species to each other. For example, the species *N. kruczakiae* and *N. veterana* and the species *N. paucivorans* and *N. brevicatena* show 99.8 and 99.5% similarity to each other, respectively (5, 25). The results of a BLAST (Basic Local Alignment Search Tool; NCBI, Bethesda, MD) analysis of the 16S rRNA gene sequences of isolates 7, 8, and 9 indicate that the next most similar type strains to these isolates are those of *N. transvalensis* and *N. wallacei*, with 98.6 and ≤98.4% similarities, respectively, which are significantly less than the similarities of these isolates to the *N. blacklockiae* type strain. As the criteria for determining conspecificity by 16S rRNA gene sequence similarity are undefined and given the DNA-DNA hybridization results, we think that all three of these isolates should be considered *N. blacklockiae* isolates.

Sequence analysis of the 16S rRNA genes of a reference strain and two patient isolates examined in this study revealed the presence of ambiguous bases that could not be resolved with repeat testing. The 16S rRNA gene of *N. wallacei* reference strain ATCC 49872 and patient isolate 9 each contained two such ambiguous bases; patient isolate 8 contained one ambiguous base. This finding suggests that these isolates may

contain multiple different copies of the 16S rRNA gene (8). The presence of ambiguous bases in the 16S rRNA gene sequence of patient isolates 8 and 9 does not appear to have been the reason for the lower level of 16S rRNA gene sequence similarity to the *N. blacklockiae* type strain; ignoring the ambiguous bases in the sequences of the patient isolates when mismatches were counted does not significantly change the percent similarity of that organism's sequence to the sequence of the type strain. One organism noted as a reference strain of *N. transvalensis* new taxon 1 (ATCC 700034) by Wilson et al. (23) contains nine ambiguous bases (data not shown) and was not included in this evaluation of members of the *N. transvalensis* complex.

There does appear to be geographic variability in the frequency of isolation of the different species included in this complex. Wilson et al. (23) noted that organisms identified as *N. transvalensis* comprised 4.2% of the *Nocardia* isolates recovered in Queensland, Australia, but only 1.1% of the isolates referred for susceptibility testing to two Texas reference laboratories. A previous study noted that 4.9% of the 102 *Nocardia* isolates from Queensland belonged to *N. transvalensis* (10). In the study of Wilson et al. (23), no isolates of *N. wallacei* and six of eight isolates (75%) of *N. blacklockiae* were isolated from Australian patients. All isolates of *N. wallacei* in the study of Wilson et al. (23) were isolated from patients in the United States.

The discrimination among the species considered to be members of the *N. transvalensis* complex is best achieved by gene sequencing methods. Phenotypic characterization with biochemicals is difficult and time-consuming, the number of tests available is small, and the data for basing species identification on phenotypic results are not robust. Initial studies that used amplification of the HSP gene and subsequent restriction endonuclease assays were able to show clear distinctions among members of this complex (23). Unfortunately, this method requires the use of numerous restriction endonucleases; it is also unclear if newly described *Nocardia* species would give RFLP patterns similar to those of members of the *N. transvalensis* complex.

The susceptibility testing results presented here illustrate some of the problems inherent in the testing of *Nocardia* species. Susceptibility testing of the same *N. wallacei* isolates in different laboratories gave various results for some drugs; if duplicate testing had been performed in each laboratory, even more variation might have been observed. In the case of the patient isolates of *N. blacklockiae*, considerable inter- and intralaboratory susceptibility test result variation was observed. It is unclear whether technical factors or differences in endpoint interpretation resulted in the variations in the susceptibility testing results. Clearly, the interpretation of endpoints with the sulfa drugs is particularly problematic; the interpretation of an 80% reduction in growth as the MIC endpoint, as is required for those drugs, is a subjective judgment and may result in disparate results.

In both case reports described above, susceptibility testing was performed in reference laboratories. It is presumed that the susceptibility testing results were not immediately available to the patients' physicians at the time of initial diagnosis and treatment. Hence, both patients received treatment with amikacin, despite the in vitro resistance of the organisms to this drug. In addition, species-related susceptibility patterns were not widely recognized at the time that treatment was initiated.

Generally, we would urge caution in assigning *Nocardia* isolates to a given species if the full 16S rRNA gene sequence differs from that of the type strain by ≤99.3%. However, given that the three patient isolates of *N. blacklockiae* (isolates 7, 8, and 9) described here have nearly identical HSP gene sequences, very similar *secA1* gene sequences, and identical SecA1 amino acid sequences to those of the type strain of *N. blacklockiae* and because the 16S rRNA gene sequences are more similar to the 16S rRNA gene sequence of *N. blacklockiae* than to the 16S rRNA gene sequences of any other species, we think they are currently best regarded as belonging to this species. In addition, DNA-DNA hybridization between each patient isolate and the type strain of *N. blacklockiae* gave RBRs of ≥85%, significantly higher than the 70% that is usually used to determine species conspecificity.

Because of its in vitro resistance to commonly used antibiotics, especially amikacin, recognition of organisms belonging to this group that are isolated from clinical specimens is particularly important and also may suggest some problems with susceptibility test performance if the susceptibility testing results deviate significantly from the results expected for these species. However, as our results with the very similar isolates investigated here show, correct species assignment of some isolates may remain problematic even after extensive study of an isolate.

Description of *Nocardia wallacei* sp. nov. *wallacei* (wal.lace′e.i, M.L. gen. masc. *wallacei*, of Wallace, in honor of Richard J. Wallace, Jr., in recognition of his contributions to the understanding of the taxonomy and drug susceptibility of *Nocardia* species). The organism is an aerobic, modified acid-fast-positive, branching gram-positive rod. Colonies produce aerial hyphae. Acid is produced from the oxidation of D-galactose, D-glucose, glycerol, maltose, salicin, sucrose, and trehalose but not from adonitol, arabinose, cellobiose, dulcitol, *i*-erythritol, D-fructose, *i-myo*-inositol, lactose, D-mannitol, mannose, melibiose, raffinose, L-rhamnose, D-sorbitol, or D-xylose. It grows at 25, 35, and 45°C, with the best growth at 35°C. It grows in the presence of lysozyme. It does not produce arylsulfatase at 3 or 14 days. It is able to hydrolyze esculin, hypoxanthine, and urea but not adenine, casein, tyrosine, or xanthine. It is not able to utilize acetamide as a sole source of carbon and nitrogen. It is able to utilize citrate (1%) as a sole source of carbon. The type strain is susceptible to ceftriaxone, ciprofloxacin, and linezolid and is resistant to amikacin and clarithromycin. The organism is a pathogen of immunocompromised patients.

The type strain of the species is ATCC 49873 (DSM 45136); and the sequences of a 1,414-bp region of the 16S rRNA gene, a 394-bp region of the HSP gene, and a 464-bp region of the *secA1* gene have been deposited in GenBank under accession numbers EU099357, EU099358, and EU099359, respectively.

Description of *Nocardia blacklockiae* sp. nov. *blacklockiae* (black.lock′.e.ae, M.L. gen. fem. *blacklockiae*, of Blacklock, in memory of Zeta M. Blacklock, in honor of and in recognition for her contributions to the study of *Nocardia* taxonomy). The organism is an aerobic, modified acid-fast-positive, branching gram-positive rod. Colonies produce aerial hyphae. Acid is produced from the oxidation of adonitol, *i*-erythritol, D-fruc-

tose, D-glucose, glycerol, maltose, D-mannitol, mannose, salicin, D-sorbitol, and trehalose but not from arabinose, cellobiose, dulcitol, D-galactose, *i-myo*-inositol, lactose, melibiose, raffinose, L-rhamnose, sucrose, or D-xylose. It grows at 25 and 35°C but not at 45°C. It grows in the presence of lysozyme. It does not produce arylsulfatase at 3 or 14 days. It is able to hydrolyze esculin, hypoxanthine, and urea but not adenine, casein, tyrosine, or xanthine. It is not able to utilize acetamide as a sole source of carbon and nitrogen. It is able to utilize citrate (1%) as a sole source of carbon. The type strain is susceptible to amoxicillin-clavulanic acid, ceftriaxone, and linezolid and is resistant to amikacin and clarithromycin. The organism is a pathogen of immunocompromised patients.

The type strain of the species is ATCC 700035 (DSM 45135); and the sequences of a 1,414-bp region of the 16S rRNA gene, a 394-bp region of the HSP gene, and a 464-bp region of the *secA1* gene have been deposited in GenBank under accession numbers EU099360, EU099361, and EU099362, respectively.

ACKNOWLEDGMENT

We thank Joann L. Cloud, ARUP Institute for Clinical and Experimental Pathology, Salt Lake City, UT; Linda Mann and Rebecca Wilson of the University of Texas Health Center at Tyler; and Xuan Qin, Children's Hospital and Regional Medical Center, Seattle, WA, for providing clinical isolates for this study. We thank Patrick R. Murray, Department of Laboratory Medicine, Warren G. Magnuson Clinical Center, NIH, for critically reviewing the manuscript.

This research was supported in part by the Intramural Research Program of the NIH Warren G. Magnuson Clinical Center.

The views expressed herein are those of the authors and should not be construed as those of the U.S. Department of Health and Human Services.

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