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Evaluation of Partial 16S Ribosomal DNA Sequencing for Identification of *Nocardia* Species by Using the MicroSeq 500 System with an Expanded Database

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Identification of clinically significant nocardiae to the species level is important in patient diagnosis and treatment. A study was performed to evaluate *Nocardia* species identification obtained by partial 16S ribosomal DNA (rDNA) sequencing by the MicroSeq 500 system with an expanded database. The expanded portion of the database was developed from partial 5′ 16S rDNA sequences derived from 28 reference strains (from the American Type Culture Collection and the Japanese Collection of Microorganisms). The expanded MicroSeq 500 system was compared to (i) conventional identification obtained from a combination of growth characteristics with biochemical and drug susceptibility tests; (ii) molecular techniques involving restriction enzyme analysis (REA) of portions of the 16S rRNA and 65-kDa heat shock protein genes; and (iii) when necessary, sequencing of a 999-bp fragment of the 16S rRNA gene. An unknown isolate was identified as a particular species if the sequence obtained by partial 16S rDNA sequencing by the expanded MicroSeq 500 system was 99.0% similar to that of the reference strain. Ninety-four nocardiae representing 10 separate species were isolated from patient specimens and examined by using the three different methods. Sequencing of partial 16S rDNA by the expanded MicroSeq 500 system resulted in only 72% agreement with conventional methods for species identification and 90% agreement with the alternative molecular methods. Molecular methods for identification of *Nocardia* species provide more accurate and rapid results than the conventional methods using biochemical and susceptibility testing. With an expanded database, the MicroSeq 500 system for partial 16S rDNA was able to correctly identify the human pathogens *N. brasiliensis*, *N. cyriacigeorgica*, *N. farcinica*, *N. nova*, *N. otitidiscaviarum*, and *N. veterana*.

Nocardia species are aerobic and saprophytic actinomycetes found in the environment. Pathogenic species have been found in dust, sand, soil and swimming pools (17). Nocardiae cause a variety of human infections including cutaneous, pulmonary, and systemic nocardiosis (17).

The well-characterized medically significant *Nocardia* species include *Nocardia abscessus* (34), *N. asteroides* (3), *N. nova* (3), *N. farcinica* (30), *N. brasiliensis* (3), *N. pseudobrasiliensis* (22, 23), *N. otitidiscaviarum* (3), and *N. transvalensis* (3, 32). The species *N. africana* (11), *N. paucivorans* (33), and *N. veterana* (5, 10, 19) have been encountered as agents of pulmonary disease (3). Other newly described *Nocardia* species have not been studied extensively for clinical relevance and include members of the *N. brevicatena* complex (3), *N. beijingensis* (31), *N. carnea* (8), *N. crassostreae* (7), and *N. vinacea* (13, 15). Members of the *N. asteroides* complex have been grouped according to drug susceptibility patterns (23, 29). *N. asteroides* drug pattern types III and V have been determined to be *N. nova* and *N. farcinica* (27, 30), respectively, while *N. asteroides* drug pattern type I has more recently been named *N. abscessus* and drug pattern type VI has been named *N. cyriacigeorgica* (21). *N. asteroides* drug pattern types II and IV have not yet been named as individual species. It is difficult to distinguish between members of the *N. asteroides* drug groups by conventional biochemical techniques; however, PCR restriction enzyme analysis (REA), as well as nucleic acid sequencing of the 16S rRNA gene, can differentiate these species (6, 21, 23).

Accurate identification of *Nocardia* species has become increasingly important as differences among species have emerged in terms of epidemiology, virulence, and antibiotic susceptibility (16). Optimal therapeutic strategies are dependent on rapid and accurate identification of *Nocardia* species including members of the *N. asteroides* complex. Molecular methods for identification, such as PCR REA and sequencing, offer a time-saving alternative to conventional methods involving growth characteristics, colony and microscopic morphology, and biochemical and susceptibility testing. We evaluated a method of sequencing base positions 4 to 532 of 16S ribosomal DNA (rDNA), corresponding to the numbering system of *Escherichia coli* 16S rDNA positions (20), by comparing the results to those obtained by conventional methods as well as

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TABLE 1. Reference strains of *Nocardia* species included in the expanded MicroSeq 500 library

Nocardia species	Reference isolate(s)
N. asteroides	ATCC 19247^T
N. abscessus[a,b]	ATCC BAA-290^T
N. asteroides drug group IV[a]	ATCC 49872, 49873
N. cyriacigeorgica[a,c]	ATCC 14759[d]
N. africana[a]	ATCC BAA-280^T
N. beijingensis[a]	JCM 10666^T
N. brasiliensis	ATCC 19296^T
N. brevicatena	ATCC 15333^T, ATCC 15725, ATCC 15726
N. carnea	ATCC 6847^T
N. corynebacteroides	ATCC 14898^T
N. crassostreae[a]	ATCC 700418^T
N. farcinica	ATCC 3318^T, ATCC 3308,[d] ATCC 23825, ATCC 23826
N. nova	ATCC 33726^T
N. otitidiscaviarum	ATCC 14629^T
N. paucivorans[a]	ATCC BAA-278^T
N. pseudobrasiliensis	ATCC 51512^T
N. seriolae	ATCC 43993^T
N. transvalensis	ATCC 6865^T, 29982
N. veterana[a]	JCM 11307^T, ATCC 33727[e]
N. vinacea[a]	JCM 10988^T

[a] Species excluded from the MicroSeq 500 database.
[b] Synonym, *N. asteroides* drug pattern type I.
[c] Synonym, *N. asteroides* drug pattern type VI.
[d] Deposited as *N. asteroides.*
[e] Deposited as *N. nova*.

alternative molecular techniques performed at the National Institutes of Health (NIH). The MicroSeq 500 system (Applied BioSystems, Foster City, Calif.) uses automated sequencing with kit-based reagents to allow analysis of 529 bases of the 16S rRNA gene (24) (partial 16S rDNA). Previous experience revealed that the MicroSeq 500 sequence library is limited to a few type strains, prompting us to develop an improved library which includes more strains of clinically significant species (4). A study was done to evaluate the accuracy of partial 16S rDNA sequencing using the MicroSeq 500 16S rDNA sequencing system with our expanded library and to compare it to other well-established conventional and molecular methods for identification of *Nocardia* species.

(Portions of this study were previously presented [J. L. Cloud, A. Croft, P. S. Conville, F. G. Witebsky, R. Yih, H. Chun, M. Martin, S. Ohlson, S. Hegewald, and K. C. Carroll, Abstr. 102nd Gen. Meet. Am. Soc. Microbiol., abstr. C-251, 2002].)

MATERIALS AND METHODS

Bacterial isolates. Nocardiae evaluated for this study were isolates recovered from clinical specimens received by the ARUP Microbiology Laboratory. All gram-positive isolates with growth characteristics and colony morphology consistent with *Nocardia* were included in this 1-year study. A total of 94 nocardia isolates were acquired; only 1 isolate was accepted per patient. Reference strains (Table 1) were purchased from the American Type Culture Collection (ATCC) and the Japanese Collection of Microorganisms (JCM).

Expanded sequencing library. The biochemical characteristics of purchased strains (identified by ATCC or JCM) were verified by conventional techniques (described below). Sequences were generated using the MicroSeq 500 system (described below) and stored in a file within the MicroSeq software package. To verify the correct species assignment of the reference strains, text files of the sequences were compared to the currently unpublished *Nocardia* database of RIDOM (12) (www.ridom-rdna.de), created and maintained by the laboratory of one of the coauthors of this paper (D.H.).

MicroSeq 500 System. DNA was extracted from organisms in pure culture by using the PrepMan Ultra reagent (Applied BioSystems) and frozen at −20°C until used in the analysis (1 to 3 days). For PCR and sequencing, the DNA samples were thawed and previously described procedures were used (4). Sequences were analyzed using the MicroSeq software system (version 1.4.3) with the MicroSeq library version 500-0125 in conjunction with our expanded library (Table 1). Eleven *Nocardia* species, listed in Table 1, are currently included in the MicroSeq 500–0125 library, while 9 species were added separately. For the purposes of this study, samples having 99.0 to 100% similarity to a species in the expanded MicroSeq 500 library were assigned that species designation provided that the colony morphology and growth characteristics were consistent. Organisms with less than 99.0% similarity to any species in the libraries were considered unidentified to the species level.

Establishing reporting criteria. The region from base positions 4 to 532 (corresponding to *E. coli* 16S rDNA positions) of the 16S rDNA were analyzed for 20 species of *Nocardia* (Table 1). Sequences were aligned, and a pairwise distance matrix was calculated using the CLUSTAL W program with the "correct for multiple substitutions" and "exclude positions with gap" options turned off (25). A Kolmogorov-Smirnov normality test was used to test the goodness of fit to a normal distribution of all pairwise distances. The Stat View version 5.0 statistical software package was used to calculate the K-S normality test, all descriptive statistical parameters, and the areas under the curve of a normal distribution (SAS Institute Inc., Cary, N.C.).

Conventional identification. *Nocardia* strains were phenotypically characterized by determination of lysozyme resistance, aerial hypha production, substrate decomposition (casein, tyrosine, xanthine, and hypoxanthine), drug susceptibility patterns, and other classical biochemical tests. Unless otherwise stated, all media were purchased from Midwest Medical (Salt Lake City, Utah). Well-isolated colonies grown on sheep blood agar or buffered charcoal yeast extract agar were inoculated to various test media as follows: (i) lysozyme and glycerol broths, (ii) substrate decomposition agar, and (iii) tap water agar (ARUP Reagent Lab, Salt Lake City, Utah). All media were incubated at 30°C and observed daily for 10 to 28 days. The results of the lysozyme resistance and substrate decomposition tests were interpreted as specified by the manufacturer's instructions for the use of lysozyme broth and degradation agar (Becton, Dickinson and Co., Sparks, Md.). If a particular isolate did not thrive or produce aerial hyphae at 30°C, the plates were transferred to a 37°C incubation to enhance growth and hypha production. The results with the test media were interpreted using the algorithm in Fig. 1. Susceptibility testing was performed either by broth microdilution (2) or by disk diffusion (28). Broth microdilution was performed only if a drug susceptibility panel was specifically requested by the physician. When no susceptibility panel was requested by the physician, drug susceptibilities were determined by nonstandardized disk diffusion methods for the sole purpose of organism identification. The drugs tested were amikacin, amoxicillin-clavulanate, ampicillin, cefotaxime, ciprofloxacin, erythromycin, imipenem, minocycline, sulfamethoxazole, and tobramycin. Interpretations were made at 24 to 48 hs. When indicated (Fig. 1), additional tests were performed by previously published methods; they included starch hydrolysis (9, 18), 14-day arylsulfatase (14), growth at 45°C (30), acetamide hydrolysis (30), acid production from rhamnose (30), and cefamandole resistance (2).

Alternative molecular techniques. Organisms grown on Trypticase soy agar were sent at room temperature to the Microbiology Service of the Warren G. Magnuson Clinical Center, National Institutes of Health. All procedures, including DNA extraction, amplification of a portion of the 16S rRNA gene, amplification of a portion of the *hsp65* gene, REA, sequence determination, and sequence analysis were performed as previously described (6). REA results for both amplicons from the 16S rRNA and *hsp65* genes were first analyzed. If discrepant REA identifications were obtained from the two gene regions, sequencing of a 999-bp portion of the 16S rRNA gene was performed and the sequences were analyzed using a BLAST search of the GenBank database (www.ncbi.nlm.nih.gov). An isolate was assigned to a particular species if the 16S rRNA sequence of the isolate was ≥99.3% similar to the type strain of the most closely related species indicated by the BLAST search. The 99.3% similarity cutoff was arbitrarily selected based on previous experience with *Nocardia* species (6). The percent similarity was determined using alignments of the sequence of the isolate in question (Megalign; DNASTAR, Madison, Wis.) with the sequence of the type strain determined in the NIH laboratory or with GenBank sequences determined to be reliable. Isolates were designated "*Nocardia* species" if the percent similarity to the type strain of the closest species was <99.3% or if the BLAST search indicated closest similarity to an unnamed species.

RESULTS

The sequences generated for the reference strains by the MicroSeq 500 system for use in the expanded database showed

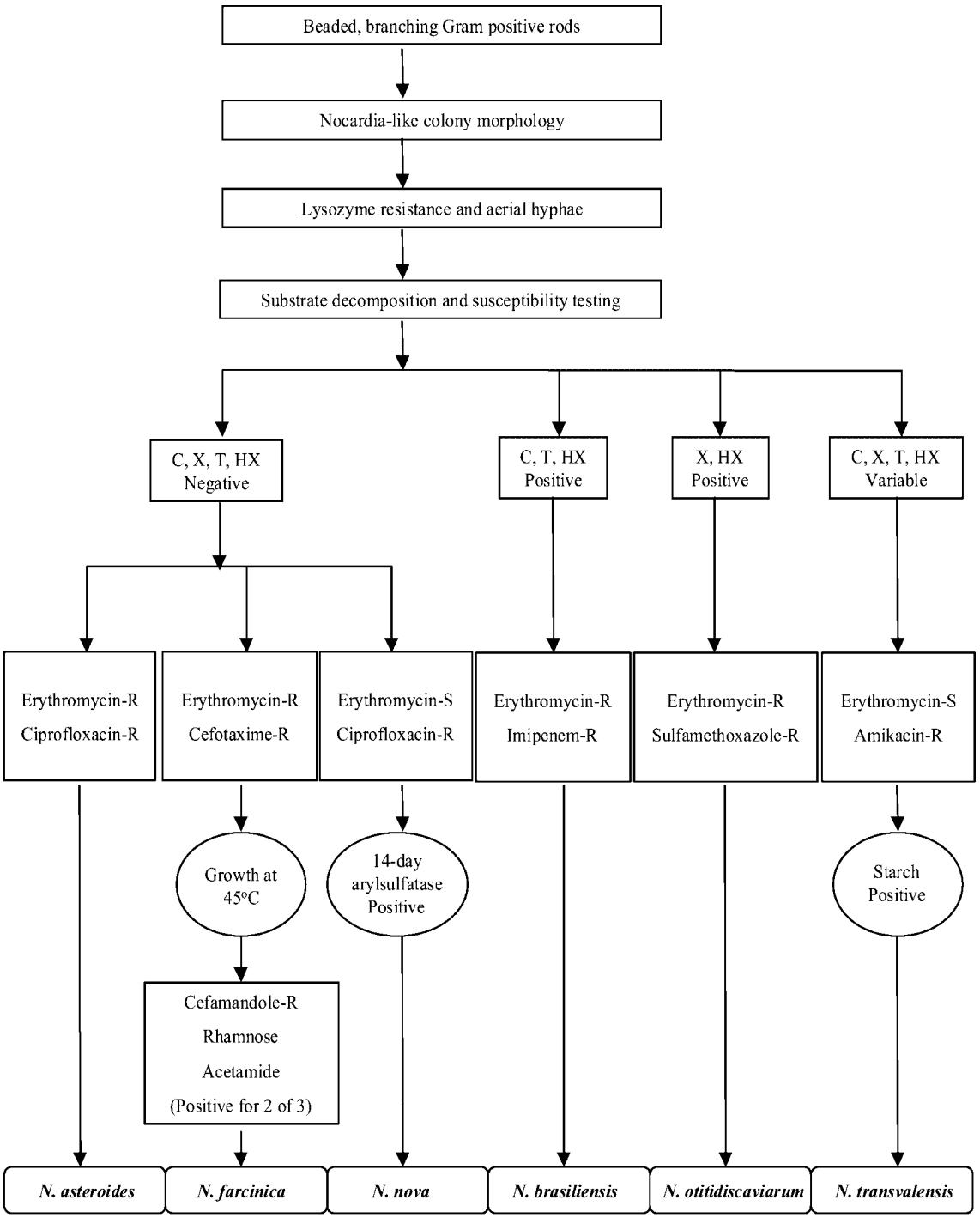

FIG. 1. Phenotypic characterization of *Nocardia* species using lysozyme resistance, aerial hypha production, substrate decomposition, susceptibility patterns, and other classical biochemical tests. R, resistant; S, sensitive; C, casein; X, xanthine; T, tyrosine; HX, hypoxanthine.

100% similarity to the sequences for these strains in the unpublished *Nocardia* database of RIDOM. A total of 94 presumptive *Nocardia* spp. were isolated and/or submitted for identification from a variety of specimen types. Table 2 summarizes the results of all comparative methods used for identification. Of a reported 31 validly named species (Deutsche Sammlung von Mikroorganismer and Zellkulturen GmbH Bacterial Nomenclature Up-to-Date [http://www.dsmz.de/bactnom/bactname.htm]), we isolated 10 distinct *Nocardia* species from clinical specimens over a 1-year period. Although the human pathogens *N. africana*, *N. paucivorans*, and *N. pseudobrasiliensis* were included in the expanded database, no clinical isolates of these species were identified by the expanded MicroSeq 500 system. The identification of *N. cyriacigeorgica* was assigned to 18 clinical isolates with 100% correlation between the expanded MicroSeq 500 system and the

TABLE 2. Isolates of 94 *Nocardia* species identified by the expanded MicroSeq 500 system and two additional identification strategies[a]

Sequence similarity[b] for MicroSeq 500 + expanded library (no. of isolates) [closest match]	Identity obtained by:	
	Alternative molecular methods[c] (no. of isolates) [technology]	Conventional methods[c] (no. of isolates)
100% *N. veterana* (3)	*N. veterana* (3) [REA/SQ]	*Nocardia* sp. (2) *N. nova* (1)
99.6% *N. veterana* (1)	*N. africana*/*N. veterana* (1) [REA/SQ]	*Nocardia* sp. (1)
100% *N. asteroides* IV (5)	*N. asteroides* IV (3) [REA (2), REA/SQ (1)], *Nocardia* sp. (2) [REA/SQ]	*N. asteroides* (3), *N. otitidiscaviarum* (1), *Nocardia* sp. (1)
99.8% *N. asteroides* IV (2)	*N. asteroides* IV (1) [REA], *Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (2)
99.6% *N. asteroides* IV (1)	*N. asteroides* IV (1) [REA]	*N. asteroides* (1)
100% *N. cyriacigeorgica* (18)	*N. cyriacigeorgica* (18) [REA (17), REA/SQ (1)]	*N. asteroides* (18)
99.8% *N. abscessus* (2)	*N. beijingensis* (2) [REA/SQ]	*N. asteroides* (1), *Nocardia* sp. (1)
100% *N. abscessus* (4)	*N. abscessus* (2) [REA/SQ] *N. beijingensis* (2) [REA/SQ]	*N. asteroides* (2) *Nocardia* sp. (2)
99.6% *N. abscessus* (2)	*N. abscessus* (2) [REA/SQ]	*N. asteroides* (1) *Nocardia* sp. (1)
100% *N. beijingensis* (3)	*N. beijingensis* (3) [REA/SQ]	*N. asteroides* (3)
99.2% *N. beijingensis* (1)	*N. beijingensis* (1) [REA/SQ]	*N. asteroides* (1)
100% *N. brasiliensis* (3)	*N. brasiliensis* (3) [REA]	*N. brasiliensis* (3)
99.8% *N. brasiliensis* (1)	*N. brasiliensis* (1) [REA]	*N. brasiliensis* (1)
99.2% *N. brasiliensis* (1)	*N. brasiliensis* (1) [REA/SQ]	*Nocardia* sp. (1)
100% *N. farcinica* (14)	*N. farcinica* (14) [REA]	*N. farcinica* (12), *N. asteroides* (1), *Nocardia* sp. (1)
100% *N. nova* (8)	*N. nova* (8) [REA (6), REA/SQ (2)]	*N. nova* (7) *Nocardia* sp. (1)
99.6% *N. nova* (8)	*N. nova* (8) [REA (7), REA/SQ (1)]	*N. nova* (8)
100% *N. otitidiscaviarum* (2)	*N. otitidiscaviarum* (2) [REA/SQ]	*N. otitidiscaviarum* (2)
99.4% *N. vinacea* (1)	*N. vinacea* (1) [REA/SQ]	*N. transvalensis* (1)
99.6% *N. vinacea* (1)	*N. vinacea* (1) [REA/SQ]	*N. asteroides* (1)
98.8% *Nocardia* sp. (1) [Ncar]	*Nocardia* sp. (1) [REA/SQ]	*N. asteroides* (1)
98.8% *Nocardia* sp. (1) [Ncar]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
98.8% *Nocardia* sp. (1) [Nbei]	*N. beijingensis* (1) [REA/SQ]	*Nocardia* sp. (1)
98.4% *Nocardia* sp. (1) [Npse/Noti/Nnov]	*N. pseudobrasiliensis* (1) [REA]	*Nocardia* sp. (1)
98.4% *Nocardia* sp. (1) [Ntra]	*Nocardia* sp. (1) [REA/SQ]	*N. asteroides* (1)
98.4% *Nocardia* sp. (1) [Nbre]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
98.3% *Nocardia* sp. (1) [Nast]	*Nocardia* sp. (1) [REA/SQ]	*N. asteroides* (1)
98.2% *Nocardia* sp. (1) [Noti]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
98.2% *Nocardia* sp. (1) [Nfar]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
98.2% *Nocardia* sp. (1) [Nafr]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
98.2% *Nocardia* sp. (1) [Nast/Nvet]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)
97.5% *Nocardia* sp. (1) [Ncar]	*Nocardia* sp. (1) [REA/SQ]	*N. asteroides* (1)
96.8% *Nocardia* sp. (1) [Nbra]	*Nocardia* sp. (1) [REA/SQ]	*Nocardia* sp. (1)

[a] Abbreviations: Nafr, *N. africana*; Nast, *N. asteroides*; Nbei, *N. beijingensis*; Nbra, *N. brasiliensis*; Nbre, *N. brevicatena*; Ncar, *N. carnea*; Nfar, *N. farcinica*; Nnov, *N. nova*; Noti, *N. otitidiscaviarum*; Npau, *N. paucivorans*; Npse, *N. pseudobrasiliensis*; Ntra, *N. transvalensis*; Nvet, *N. veterana*; REA, restriction endonuclease assay performed for portions of both the 16S rRNA gene and the heat shock protein gene; REA/SQ, restriction endonuclease assay and 16S rRNA gene sequencing performed.

[b] Similarity score refers to the expanded MicroSeq 500 system.

[c] See the text for a description.

molecular methods used by the NIH laboratory. These were identified as *N. asteroides* by the phenotypic methods.

There were 14 *N. farcinica* and 16 *N. nova* species identified with excellent correlation among the molecular methods. By conventional methods, one isolate each of *N. farcinica* and *N. nova* could not be definitively identified to species level and another *N. farcinica* isolate was identified as *N. asteroides.* Identification of *N. brasiliensis* and *N. otitidiscaviarum* correlated well among the three methods. Two strains of *N. vinacea* identified as such by all sequencing methods were identified as *N. transvalensis* and *N. asteroides* by conventional methods.

N. africana (11) and *N. veterana* (10) are two recently described species that are phylogenetically related and phenotypically similar to each other (5). Reference strains of *N. africana* (ATCC BAA-280^T) and *N. veterana* (JCM 11307^T) were sequenced using the expanded MicroSeq 500 system for comparison with four isolates with sequence similarity to *N. africana* and *N. veterana.* Partial 16S rDNA sequences from three of the four isolates showed 100% similarity to the type strain of *N. veterana* when the expanded MicroSeq 500 system was used. The remaining isolate was also identified as *N. veterana* with a similarity score of 99.6%. Alternative molecular techniques identified three of these isolates as *N. veterana* and one as belonging to the *N. africana-N. veterana* group. Using the expanded MicroSeq 500 system, all of the *N. veterana* clinical isolates were 99.0% similar to the *N. nova* type strain (ATCC 33726). None of the clinical isolates matched the *N. africana* type strain. The reference strain of *N. nova* (ATCC 33727) showed 100% similarity to *N. veterana* by the expanded MicroSeq 500 system, suggesting that this strain is actually *N. veterana* and was deposited with ATCC before *N. veterana* was described.

Three isolates showed 100% similarity by the expanded MicroSeq 500 system to the sequence of the type strain of *N. beijingensis*. Another isolate was 99.2% similar to *N. beijingensis*. Identification of the four isolates by the expanded MicroSeq 500 system was in agreement with identification by alternative molecular methods, but they were identified as *N. asteroides* by conventional methods.

Eight isolates were identified by the expanded MicroSeq 500

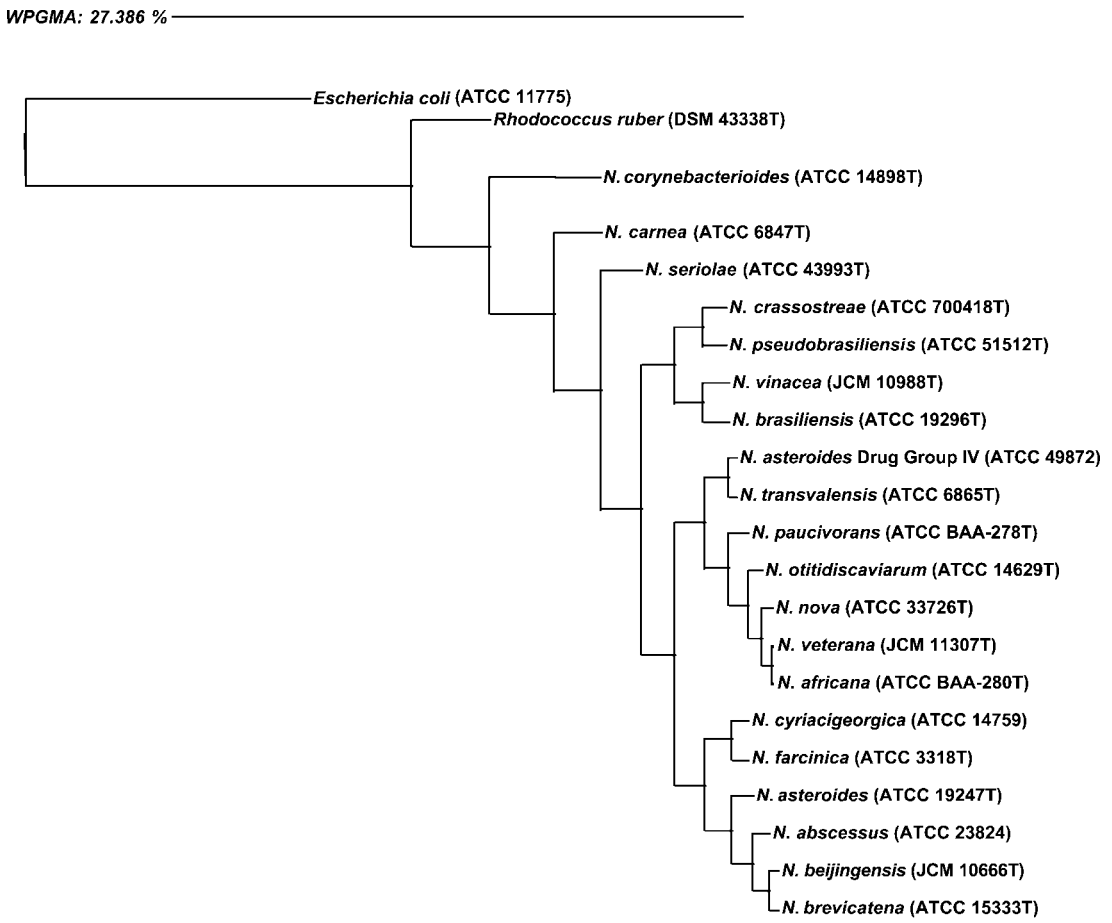

FIG. 2. Phylogenetic tree relating the partial 16S rDNA sequences included in the expanded MicroSeq 500 library. Two designated outgroups, *Escherichia coli* (ATCC 11775^T) and *Rhodococcus ruber* (ATCC 14898^T), are included for reference of relatedness. The algorithm used to construct the tree is the unweighted pair group method using averages (UPGMA). Reference strain numbers ending in T are type strains.

system as *N. asteroides* drug pattern IV. Conventional methods identified these as *N. asteroides* (four isolates), *Nocardia* sp. (three isolates), and *N. otitidiscaviarum* (one isolate). Alternative molecular methods identified these as *N. asteroides* IV/*transvalensis* (four isolates), *Nocardia* sp. (three isolates), and *N. asteroides* drug pattern type IV (one isolate).

N. abscessus shows a close phylogenetic relationship to *N. beijingensis* (Fig. 2) on the basis of partial 16S rDNA sequences. Roth et al. (21) evaluated the phylogenetic relationship by using full 16S rDNA sequences and demonstrated that *N. beijingensis* is not closely related to *N. abscessus*, suggesting further sequencing beyond the first 500 bases may be necessary for accurate delineation of the relationship between these species. Of eight isolates identified as *N. abscessus* by the expanded MicroSeq 500 system, four showed 100% similarity to the *N. abscessus* type strain, two showed 99.8% similarity to the type strain, and two were 99.6% similar to the type strain. Sequencing 999 bp of the 16S rRNA gene assigned *N. beijingensis* to half of this group and *N. abscessus* to the remaining half for 50% agreement with the expanded MicroSeq 500 system. Conventional methods named five of the isolates as *N. asteroides* and the remaining three as unassigned species.

Fourteen isolates were identified by the expanded MicroSeq 500 system as members of the *Nocardia* genus but were not assigned a definitive species because of too much divergence in sequence similarity. One isolate assigned a species designation by the alternative methods as *N. beijingensis* (99.3% similarity to the *N. beijingensis* type strain) had a similarity index by the expanded MicroSeq 500 system which was below the arbitrary cutoff. One isolate was identified as *N. pseudobrasilisensis* by REA; subsequent 16S rRNA gene sequencing of the 999-bp region to resolve this discrepancy showed this isolate to be unrelated to any described *Nocardia* species.

The mean pairwise distance of 20 unique partial rDNA nocardiae sequences was 3.2%, the standard deviation was 1.2%, the minimum was 0.2%, and the maximum distance was 6.6%. The result of a K-S normality test was not significant (i.e., $P < 0.05$), suggesting that the pairwise distances are normally distributed. Our choice of a reporting criterion of ≥99.0% similarity (1.0% divergence) for a "distinct species" correlates to a statistical error probability of 3.0% (i.e., in only 3.0% of cases do two distinct species exhibit a similarity value greater than or equal to 99.0%).

DISCUSSION

The primary aim of the present study was to assess the reliability of an expanded database for the MicroSeq 500 se-

quencing system used to identify *Nocardia* species from clinical isolates. When identifying *Nocardia* species using the expanded MicroSeq 500 system, accuracy was verified when similar results were obtained by using PCR REA of two separate gene targets and 999-bp 16S rDNA sequencing with a BLAST search of the GenBank public database (1) and subsequent independent alignments when necessary. Throughout the study, GenBank was used with caution since 16S rDNA sequences of many *Nocardia* species are not included in public databases, taxonomic changes are not often applied to public databases, and many faulty entries are found in publicly accessible databases (26).

Conventional methods for identification of *Nocardia* species produced more isolates that could not be assigned to a particular species (23%) than did the expanded MicroSeq 500 system (14%) or alternative molecular methods (15%). There were 10 discrepancies between the expanded MicroSeq 500 system and the alternative molecular methods, probably due to the number of bases examined by each technique, the use of different databases, and different interpretive strategies. Of the 10 discrepancies, 4 were identified as *N. abscessus* by the expanded MicroSeq 500 system but called *N. beijingensis* by the alternative molecular techniques. These discrepancies are a result of sequence differences in the 16S rRNA gene region not examined by the expanded MicroSeq 500 system. While all four isolates showed significant similarity to the *N. abscessus* type strain (ATCC BAA-290^T) in the first 500-bp region (99.8 to 100% similarity with 0 to 1 base difference), sequence analysis of the 999-bp region showed these isolates to be only 98.3 to 98.4% similar (14 to 15 base differences) to the *N. abscessus* type strain. Sequence analysis of the 999-bp region showed 99.3 to 99.4% similarity to the *N. beijingensis* type strain with 5 to 6 base differences. Therefore, any isolate identified as *N. abscessus* by the expanded MicroSeq 500 system should be further analyzed using a larger region of the 16S rRNA gene to provide a more definitive identification. Because *N. beijingensis* has not yet been implicated as a human pathogen, more studies are needed to determine if isolates identified as *N. beijingensis* in this study are actually members of this species and to further define *N. beijingensis*-like clinical isolates whose sequences are similar but not 100% identical to the type strain.

As with *N. beijingensis*, *N. vinacea* has not yet been implicated as a human pathogen. Further studies are necessary to determine if the isolates identified as *N. vinacea* in this study are actually representatives of this species and to determine the possible role of this species in human disease.

Three of the discrepancies involved isolates identified as *N. asteroides* drug pattern IV by the expanded MicroSeq 500 system but unidentified to the species level by the alternative molecular methods. Sequence analysis of the 999-bp region of one of these isolates showed 99.1% similarity to the *N. asteroides* drug pattern IV reference strain. By the alternative molecular methods, two isolates showed ≤98.2% similarity to the *N. asteroides* drug pattern IV reference strain; as noted above for *N. abscessus*, most of the sequence divergence for these isolates occurred in the extended gene region not examined by the MicroSeq 500 system.

The two remaining discrepancies were due to similarity scores achieved with the expanded MicroSeq 500 system of less than 99.0%. Both had the closest match as being the assigned species of the alternative molecular methods (*N. beijingensis* and *N. pseudobrasiliensis*). The isolate identified as *N. beijingensis* by alternative molecular techniques showed 99.3% similarity to the *N. beijingensis* type strain, with all base discrepancies occurring within the first 500 bases. The misidentification of *N. pseudobrasiliensis* by REA indicates a potential pitfall in using REA alone for the identification of this species, and further studies need to be performed to optimize *N. pseudobrasiliensis* identification using this method.

Nocardia isolates that could not be definitively identified to the species level are probably unidentifiable because a large number of *Nocardia* species still await recognition as new species. We expect this percentage to become smaller as new taxonomic descriptions lead to valid species. From recent reports (21), it is suggested that *N. asteroides* drug pattern type II has a partial 16S rDNA sequence identical to that of a clinical isolate in this study that was 98.8% similar to *N. carnea* (Table 2). The strain probably represents a new species and requires further genetic and phenotypic evaluation to become a valid species. With proper database management, one can add sequences of new species to a database as they become validly described and made available for studies.

It is interesting that the sequence of the *N. asteroides* type strain did not match any of the clinical isolates. Most (53%) of the *N. asteroides* complex isolates gave perfect matches to *N. cyriacigeorgica*, while the remaining isolates were split among *N. asteroides* drug group IV and *N. abscessus.*

Of 81 species assigned, 21 demonstrated greater than 99.0% but less than 100% similarity to the most closely related species when tested by the expanded MicroSeq 500 system. These could be variants of the same species or distinct but closely related species, or could represent an error in base sequence analysis. For the present study, the chance of incorrect base sequence analysis was low, since we used sequence analysis by two independent individuals and any sequence requiring more than 10 edits was more closely evaluated and repeated, if necessary.

The question of how similar a strain must be to a reference strain before a species should be assigned by the expanded MicroSeq 500 system was addressed via statistics, but the assignment is arbitrary and still requires confirmation by further molecular analysis including DNA-DNA hybridization. Based on our experience with *Mycobacterium* species, we established reporting criteria for partial 16S rDNA sequence analysis to report a species if it is greater than 99.0% similar to the reference strain in the database. Calculations for reporting a threshold will vary among genera due to the varying diversity exhibited in each genus. As our database grows, with the addition of new species and sequevars of the same species, calculation of the reporting threshold will change. Ideally, all *Nocardia* species possible should be included in the calculation of reporting criteria.

The most important component for successful sequencing in the identification of bacterial isolates is an accurate and complete database. In the era of 16S rDNA sequencing, we continue to find more changes being made to *Nocardia* taxonomy as well as numerous descriptions of new species. A large collaborative effort to establish a 16S rDNA database would contribute significantly to the success of clinical laboratories in bringing 16S rDNA sequencing into their bacterial identifica-

tion algorithms. Such collaborations may also lead to (i) development of future recommendations for reporting criteria, (ii) assessment of the clinical significance of new species, (iii) recommendations of when and how to distinguish certain species possessing identical 16S rDNA sequences, and (iv) the development of quality assurance protocols. A high-quality, complete, well-maintained, freely accessible 16S rDNA database would allow many laboratories further advancement in bacterial identification.

Once an improved database was developed, the incorporation of the expanded MicroSeq 500 system into our identification algorithm brought more accuracy to the identification of *Nocardia* species while significantly decreasing the turnaround time from that of conventional identification. The average time to identify *Nocardia* isolates in culture has dropped from 2–3 weeks to 1–3 days when expanded MicroSeq 500 sequencing is employed.

This study shows that the expanded MicroSeq 500 system is able to identify six human pathogens (*N. brasiliensis*, *N. cyriacigeorgica*, *N. farcinica*, *N. nova*, *N. otitidiscaviarum*, and *N. veterana*). *N. abscessus* and *N. asteroides* drug pattern IV are not distinctly identified but are easily differentiated from other species, and for these species extended regions of the 16S rDNA gene can be analyzed to give a more accurate species assignment. The ability of the system to identify the human pathogens *N. africana*, *N. paucivorans*, and *N. pseudobrasiliensis* has not been determined.

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