

Clinical and Laboratory Features of the *Nocardia* spp. Based on Current Molecular Taxonomy

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INTRODUCTION

Although some controversy exists concerning the specific number of valid species of *Nocardia*, the genus *Nocardia* currently contains more than 50 species that have been character-

ized by phenotypic and molecular methods (Tables 1 and 2) (56). However, not all of these species have been subjected to the same level of analysis. Further work may reveal that a few of these species are not indeed “valid.” Among the currently accepted species, approximately half are recognized human and/or animal pathogens. Manifestations of disease range from cutaneous infection caused by traumatic inoculation of the organism in a normal host to severe pulmonary or central nervous system (CNS) disease in an immunocompromised host (9, 20, 22, 24, 61, 171, 204).

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TABLE 1. Current validly described *Nocardia* species of human clinical significance

Species	Reference(s)	Commonly associated disease ^a		
		Primary skin	Pulmonary	Disseminated
<i>N. abscessus</i>	228		+	+
<i>N. africana</i>	82		+	
<i>N. anaemiae</i>	101			
<i>N. aobensis</i>	95			
<i>N. araoensis</i>	98			
<i>N. arthritis</i>	96			
<i>N. asiatica</i>	92		+	
<i>N. asteroides</i>	54	+	+	+
<i>N. beijingensis</i>	93, 217		+	+
<i>N. brasiliensis</i> (formerly <i>Discomyces brasiliensis</i> , <i>Streptothrix brasiliensis</i> , <i>Oospora brasiliensis</i> , <i>Actinomyces brasiliensis</i>)	125	+		
<i>N. brevicatena</i> (formerly <i>Micropolyspora brevicatena</i>)	122			
<i>N. carneae</i>	166			
<i>N. cyriaciageorgica</i> ^b (formerly <i>N. cyriaciageorgici</i>)	229		+	+
<i>N. farcinica</i>	189		+	+
<i>N. higoensis</i>	97		+	+
<i>N. inohanensis</i>	99			
<i>N. kruczakiae</i>	41			
<i>N. mexicana</i> ^c	165			
<i>N. niigatensis</i>	94	+		
<i>N. nova</i>	192		+	+
<i>N. otitidiscaviarum</i> (formerly <i>N. caviae</i>)	179	+	+	+
<i>N. paucivorans</i>	227		+	+
<i>N. pseudobrasiliensis</i>	168		+	+
<i>N. pneumoniae</i>	98			
<i>N. puris</i>	231			
<i>N. sienata</i> (formerly <i>N. senatus</i>)	100			
<i>N. testaceae</i> (formerly <i>N. testaceus</i>)	100			
<i>N. thailandica</i>	94			
<i>N. transvalensis</i>	160		+	+
<i>N. vermiculata</i>	94			
<i>N. veterana</i>	80		+	
<i>N. vinacea</i>	101, 109			
<i>N. yamanashiensis</i>	99			

^a Four or more known cases.

^b The original spelling of the epithet in *N. cyriaciageorgici* [sic] has been corrected by the List Editor, IJSEM (126).

^c Not validated.

The species of the genus *Nocardia* in the family *Nocardiaceae* form a homogenous cluster among the *Corynebacteriaceae*, a suborder of the order *Actinomycetales*. The present and generally accepted definition of *Nocardia* is based mainly on molecular phylogenetic information, notably that from 16S rRNA gene sequences (180). Like members of the genus *Mycobacterium*, the nocardiae contain tuberculostearic acids, but in contrast to mycobacteria, they also possess short-chain (40- to 60-carbon) mycolic acids and usually exhibit characteristic branching on Gram staining. The nocardiae also have a peptidoglycan cell wall composed of meso-diaminopimelic acid, arabinose, and galactose (70, 71, 72, 73, 74, 121, 145, 146, 160).

Nocardiae are ubiquitous in the environment and can be found worldwide as saprophytic components in fresh- and salt-water, soil, dust, decaying vegetation, and decaying fecal deposits from animals (8, 20, 50, 66, 70, 79, 117, 198, 224). Some species are more prevalent in geographical locations with a specific climate. For example, *Nocardia brasiliensis*, usually associated with cutaneous infection and mycetoma, is more com-

TABLE 2. Current validly described *Nocardia* species from the environment and from aquatic hosts

Species	Reference
<i>N. alba</i>	124
<i>N. caishijiensis</i>	232
<i>N. cerradoensis</i>	2
<i>N. coeliaca</i>	79
<i>N. crassostreae</i> ^a	65
<i>N. cummidelens</i>	133
<i>N. flavorosea</i>	35
<i>N. fluminea</i>	133
<i>N. ignorata</i>	230
<i>N. neocaledoniensis</i>	169
<i>N. pigrifrangens</i>	216
<i>N. pseudovaccinii</i> (formerly <i>N. vaccinii</i>).....	107
<i>N. salmonicida</i> (formerly <i>Streptomyces salmonica</i>) ^a	87, 133
<i>N. seriola</i> ^a (formerly <i>N. seriolae</i>).....	116
<i>N. shimofusensis</i>	97
<i>N. soli</i>	133
<i>N. takedensis</i>	225
<i>N. tenerifensis</i>	103
<i>N. uniformis</i>	88
<i>N. vaccinii</i>	49
<i>N. xishanensis</i>	233

^a Causes disease in aquatic hosts.

monly isolated in areas with tropical or subtropical climates and is often encountered in the southwestern or southeastern United States (24, 60, 206).

Saubolle and Sussland recently reported that nocardial infections in the United States seem to be more prevalent in the arid, warm climates of the southwest. They hypothesize that the dry, dusty, and often windy conditions in these areas may facilitate the aerosolization and dispersal of the nocardiae and thus enhance their acquisition via inhalation of the fragmented cells (173).

Health care-associated transmission or acquisition of the nocardiae has been documented but is relatively rare. However, nocardial infections are not considered to be communicable from person to person, although this may relate to the relative infrequency of close association of high-risk patients (84).

In this review, we provide an overview and an update on the taxonomy of the *Nocardia* spp. and point out many of the changes brought about by newer molecular technologies used for species identification. These include methodologies such as 16S rRNA gene sequencing and PCR restriction fragment length polymorphism (RFLP) analysis of both the 65-kDa heat shock protein-encoding gene (*hsp65*) and the 16S rRNA gene (43, 181, 183, 186). We also address clinical disease caused by the nocardiae, including health care-associated disease, and the need for antimicrobial susceptibility testing for effective drug treatment. The crucial taxonomic issue of the nomenclature of "*Nocardia asteroides*" (the type species of the genus) is also addressed.

HISTORY OF NOCARDIA

The taxonomic history of the genus *Nocardia* is fraught with confusion and controversy. The organism initially placed in the genus *Nocardia* was isolated by veterinarian Edmond Nocard in 1888 from a case of bovine farcy (lymphadenitis) (153). One year later, Trevisan characterized the organism and named it

Nocardia farcinica (189). In 1954, *N. farcinica* was made the type species of the genus *Nocardia*, and this strain was designated the type strain of the species *N. farcinica*. Examples of the original strain, known as *Nocardia farcinica* Trevisan, were maintained in two different national culture collections. Unfortunately, subsequent phenotypic studies showed two markedly different organisms; one strain was characterized as a *Mycobacterium* strain (NCTC 4524) and the other as a *Nocardia* strain (ATCC 3318^T) (31, 120, 189). Taxonomic studies by Gordon and Mihm in 1962 found no phenotypic differences between ATCC 3318 and strains of isolates known as "*N. asteroides*" (75, 76, 77, 163). Because of the uncertain taxonomic status of the *N. farcinica* type strain and because *N. asteroides* had become the most common designation of isolates of this genus, Gordon and Mihm petitioned the International Judicial Commission to group these two species together; the name *N. asteroides* was chosen to replace *N. farcinica* as the type species of the genus *Nocardia*. At this time, a new type strain, *N. asteroides* ATCC 19247, was also selected (75, 76, 120).

Although it is not known which of the "*N. farcinica*" strains in the two culture collections is representative of Nocard's original organism, ATCC 3318^T possesses all the characteristics of a *Nocardia* strain, and subsequent studies, beginning with Tsukamura in 1969 and continuing with other investigators, have shown that collections of organisms considered to belong to *N. asteroides* contained a subgroup of isolates that were biochemically and immunologically the same as ATCC 3318^T but not ATCC 19247^T (19, 64, 117, 132, 163, 190, 191, 192, 193, 194, 196). Because of these findings, the International Judicial Committee continued to use the name *N. farcinica*, although the species distinction between *N. farcinica* and *N. asteroides* remained controversial. Recently, with the application of methods including susceptibility testing and molecular studies, the species status of *N. farcinica* was clearly established as separate from *N. asteroides*.

A major taxonomic and nomenclature-related issue for *Nocardia* is the status of the name "*Nocardia asteroides*" and the fact that many (if not all) of the isolates reported in the literature as "*N. asteroides*" were clearly misidentified by today's standards. This species was chosen by Ruth Gordon as the type species of *Nocardia*, with ATCC 19247 as the type strain of the type species. Because molecular testing was not yet available and because members of the species "*N. asteroides*" are relatively inert biochemically, this organism was not easily characterized. Unfortunately, subsequent 16S rRNA gene sequencing of the type strain and clinical isolates of *Nocardia* failed to show the sequence of the type strain among these clinical isolates. Hence, unless the name "*Nocardia asteroides*" is assigned to another molecularly identified group, the epithet of "*N. asteroides*" will gradually disappear and will no longer represent the usual isolate most frequently identified in cases of human nocardiosis. Previous studies from the southern United States have shown that the most common group within the older definition of *N. asteroides* (based on the absence of hydrolysis of casein, tyrosine, and xanthine) is the group that Wallace et al. (213) designated the type VI drug pattern. In that study by Wallace et al., the type VI drug pattern represented approximately 35% of clinical isolates of *N. asteroides*, and it might be a logical choice to become the "new" *N. asteroides* (213, 214). Isolates of this group were recently found

to be identical in 16S rRNA gene sequence to the newly described species *N. cyriacigeorgica* (229). Although Yassin et al. named the organism *Nocardia cyriacigeorgici*, the species name was changed by the list editor at the *International Journal of Systematic and Evolutionary Microbiology* (126) to *N. cyriacigeorgica*, which is proposed as being etymologically correct. The issue of the disposition of the epithet *N. asteroides* and the implications of assigning this name to another species or drug pattern group will require assessment by the International Judicial Committee.

TAXONOMY OF *NOCARDIA*

Nocardia asteroides Complex

Historically, *N. asteroides* was defined biochemically as including those isolates of *Nocardia* that do not decompose xanthine, tyrosine, and casein (78). In 1951, Gordon, Mishra, and colleagues began studying five species within the genus *Nocardia*, including *N. carnea*, *N. vaccinii*, *N. transvalensis*, and two other species that were later transferred to other genera. These five groups of strains were compared to the nine species of *Nocardia* recognized at that time by using phenotypic and physiological characteristics such as colonial morphology, hydrolysis reactions, biochemicals, and chromatographic determinations of whole-cell hydrolysates of representative strains (75, 76, 77, 78). Subsequently, the investigators devised a key for the tentative identification of species of *Nocardia*, leading to the identification of many isolates of medical importance (144).

In 1988, Wallace and colleagues (213) reported six drug pattern types among a study of 78 clinical isolates previously identified as "*Nocardia asteroides*." This was the largest study of antimicrobial susceptibilities of *N. asteroides* to show the variability of drug susceptibility patterns and the first to show grouping of specific susceptibility patterns. The issue of the type strain was first evident in this study, as none of the six drug pattern groups matched the pattern of the type strain ATCC 19247. This study provided the impetus for investigators to perform studies of drug resistance in *N. asteroides* (subsequently referred to as the *N. asteroides* complex) and for the study of the taxonomic significance of the groups of *Nocardia* exhibiting the six drug susceptibility patterns. These later studies were performed using more modern molecular methods (Table 3).

Detailed studies of isolates with the six individual *N. asteroides* drug patterns followed the initial 1988 report, and these new studies described several of the drug patterns as distinct species/taxa of *Nocardia*. These groups included isolates with the type I drug susceptibility pattern, which have been noted as being identical to isolates defined as *N. abscessus* (213, 228); isolates with the type III drug susceptibility pattern, which were later shown to be a complex of organisms related to *N. nova* (41, 44, 45, 192, 207, 213, 226); isolates with the type V drug susceptibility pattern, which were subsequently shown to be *N. farcinica* (213, 214); and isolates with the type VI drug susceptibility pattern, which have recently been reported to be identical to *N. cyriacigeorgica* (157, 213, 229).

The type II drug pattern has yet to receive a species designation but has been presumptively associated with *N. brevicatena* and *N. paucivorans* in the *N. brevicatena/paucivorans* complex, as well as with some isolates of *N. carnea* and other *Nocardia* species in

TABLE 3. Antimicrobial susceptibility patterns of species of the former *Nocardia asteroides* complex, *N. brasiliensis*, *N. pseudobrasiliensis*, and *N. otitidiscaviarum*

Species	Corresponding type drug pattern	Major drug pattern characteristics
<i>N. abscessus</i>	I	Susceptible to ampicillin, amoxicillin-clavulanic acid, ceftriaxone, linezolid, and amikacin; most have resistant MICs for imipenem; resistant to ciprofloxacin and clarithromycin
<i>N. brevicatena/paucivorans</i> complex, unnamed group	II	Same as type I but kanamycin MICs low (<1 µg/ml) and susceptible to ciprofloxacin; usually resistant to gentamicin; resistant to clarithromycin
<i>N. nova</i> complex (<i>N. nova</i> , <i>N. veterana</i> , <i>N. africana</i> , <i>N. kruczakiae</i>)	III	Susceptible to ampicillin but resistant to amoxicillin-clavulanic acid; susceptible to erythromycin, clarithromycin, linezolid, and ceftriaxone; very low MICs to imipenem and amikacin
<i>N. transvalensis</i> complex	IV	Resistant to all aminoglycosides, including amikacin; susceptible to ciprofloxacin, ceftriaxone, linezolid, and imipenem; resistant to erythromycin and clarithromycin
<i>N. farcinica</i>	V	Resistant to ampicillin, broad-spectrum cephalosporins, and clarithromycin; resistant to aminoglycosides except amikacin; susceptible to ciprofloxacin, linezolid, and imipenem
<i>N. asteroides</i> complex	VI	Resistant to ampicillin, amoxicillin-clavulanic acid, clarithromycin, and ciprofloxacin; susceptible to ceftriaxone, amikacin, linezolid, and imipenem
<i>N. asteroides</i> type VI (unnamed), <i>N. cyriacigeorgica</i>		The ATCC type strain is susceptible to ampicillin, other drug susceptibilities are the same as for pattern VI
<i>N. brasiliensis</i>	NA ^a	Susceptible to minocycline, amoxicillin-clavulanic acid, carbenicillin, and sulfamethoxazole; resistant to kanamycin, cefamandole, ampicillin, ciprofloxacin, and clarithromycin
<i>N. pseudobrasiliensis</i>	NA	Susceptible to carbenicillin, ciprofloxacin, clarithromycin, and sulfamethoxazole; resistant to kanamycin, cefamandole, ampicillin, minocycline, and amoxicillin-clavulanic acid
<i>N. otitidiscaviarum</i>	NA	Susceptible to kanamycin, gentamicin, amikacin, sulfamethoxazole, and ciprofloxacin; resistant to ceftriaxone, ampicillin, amoxicillin-clavulanic acid, carbenicillin, and imipenem (often resistant to all β-lactam antibiotics)

^a NA, not applicable.

generally different phylogenetic clades (157, 167, 227; B. A. Brown, R. W. Wilson, V. A. Steingrube, Z. Blacklock, and R. J. Wallace, Jr., Abstr. 97th Gen. Meet. Am. Soc. Microbiol., abstr. C-65, p. 131, 1997).

An additional miscellaneous drug pattern includes the type strain ATCC 19247 of *N. asteroides*, but no isolate molecularly identical to this strain has been identified as a cause of human disease (213).

Isolates belonging to drug susceptibility pattern type IV have yet to receive a species designation but were later included in the *N. transvalensis* complex along with several other taxonomic groups (181, 213, 223).

The following is a discussion of these characterized species or complexes as correlated to the former drug pattern types.

***Nocardia abscessus* (Formerly *N. asteroides* Type I Drug Susceptibility Pattern)**

Wallace et al. first recognized *N. abscessus* as the type I drug pattern in their 1988 study of drug susceptibility patterns within *N. asteroides* (213). These isolates exhibited a unique β-lactam susceptibility pattern of susceptibility to ampicillin, amoxicillin-clavulanic acid, carbenicillin, and the broad-spectrum cephalosporins (cefotaxime and ceftriaxone), but with high MICs (8 to 32 µg/ml) for imipenem, making it one of the few species other than *N. brasiliensis* and the *N. otitidiscaviarum* complex that are resistant to this drug (Table 3). Additionally, this species is susceptible to sulfamethoxazole, gentamicin, and amikacin; susceptible or intermediate to minocycline; and resistant to ciprofloxacin (and the other fluoroquinolones), erythromycin, and (hence presumably) clarithromycin (213).

In 1995, Steingrube and colleagues characterized six of the seven drug patterns of *N. asteroides* by PCR restriction enzyme

analysis (PRA) of a 441-bp region of the *hsp65* gene (181). They demonstrated two RFLPs for members of the type I drug pattern that were unique from those for other identified taxa. PRA with MspI showed bands of 265 and 180 bp. The majority of isolates studied had BsaHI band patterns of 270, 70, and 65 bp, whereas fewer than 20% of the isolates had a second pattern of 335 and 65 bp (181). Biochemically these isolates were found to be relatively asaccharolytic and were positive only for citrate as a sole carbon source.

In 2000, Yassin and colleagues described three clinical strains of *Nocardia* that contained a previously undescribed 16S rRNA gene sequence that showed 98.9% similarity to the 16S rRNA gene of the type strain of *N. asteroides*, ATCC 19247 (228). The three clinical strains were identical phenotypically to ATCC 23824, a former *N. asteroides* complex strain characterized by Wallace et al. as having a type I drug susceptibility pattern and also included as a member of the type I group in the *hsp65* gene PRA study by Steingrube et al. (213, 228). This taxonomic group had previously been shown to represent approximately 20% of clinical isolates grouped within the *N. asteroides* complex as determined by drug susceptibility patterns (213).

Biochemically, the three clinical strains in the study by Yassin et al. were identical to other members of the *N. asteroides* complex in that they were unable to hydrolyze the traditionally tested substrates of xanthine, tyrosine, casein, and hypoxanthine (228).

Sequencing of the *hsp65* gene to determine the number of sequence variants (sequevars) within *N. abscessus* has not been done, but PCR restriction analysis has demonstrated at least two RFLPs within this group. Sequence analysis of 1,236 bp of the 16S rRNA genes of the three clinical strains and of ATCC 23824 found them to have 100% identity (228).

DNA-DNA hybridization experiments showed that all four strains studied by Yassin et al. (three clinical strains and ATCC

23824) belonged to the same genospecies. The G+C content ranged from 67.5 to 69.8 mol % for the species which is represented by type strain DSM 44432 (228). These studies established that isolates belonging to the *N. asteroides* type I drug susceptibility group were a new species, henceforth called *N. abscessus*.

***Nocardia brevicatena/paucivorans* Complex (Includes Type II Drug Susceptibility Pattern)**

The *N. brevicatena/paucivorans* complex is composed of at least two and probably three species, including *N. brevicatena* and *N. paucivorans* and isolates designated as belonging to the former *N. asteroides* drug pattern type II. *N. brevicatena* was formerly classified as *Micropolyspora brevicatena* (40, 122) and subsequently was transferred to the genus *Nocardia* (73) based upon its cell wall composition.

The isolates of *N. asteroides* drug susceptibility pattern type II exhibited susceptibility to ampicillin, carbenicillin, ciprofloxacin, kanamycin, and broad-spectrum cephalosporins (213). A unique feature of this group was susceptibility to amikacin, kanamycin, and tobramycin but resistance to gentamicin. The isolates are relatively inert biochemically, with trehalose and/or L-rhamnose being the only carbohydrates utilized (Brown et al., Abstr. 97th Gen. Meet. Am. Soc. Microbiol.).

Isolates of *N. brevicatena*, like those of *N. paucivorans* described by Yassin et al. (227), do not decompose casein, xanthine, tyrosine, or hypoxanthine. Isolates of *N. brevicatena* and *N. paucivorans* also possess an inert carbohydrate pattern, with utilization of only trehalose as a sole source of carbon. *N. brevicatena* differs from *N. paucivorans* only in its utilization of isoamyl alcohol and 1,2-propanediol (227).

In their description of *N. paucivorans*, Yassin et al. (227) reported that *N. brevicatena* and *N. paucivorans* show 99.6% 16S rRNA gene sequence similarity (in a nearly 1,400-bp region). However, the results of DNA-DNA hybridization studies and phenotypic testing indicate that they are distinct species (227). By partial 16S rRNA sequencing (500 bp), five clinical isolates of *N. asteroides* drug pattern type II were most closely related to the type strain of *N. carnea* in the MicroSeq database, with similarities of 99.6 to 99.8%. At that time it did not appear that reference strains of *N. asteroides* drug pattern II were included in the MicroSeq database (157).

***Nocardia nova* Complex (Formerly *N. asteroides* Type III Drug Susceptibility Pattern and Including *N. africana*, *N. kruczakiae*, *N. nova*, *N. veterana*, and Unnamed Species)**

Tsukamura first described *N. nova* in 1982 (192) as a separate species, distinct from *N. asteroides* and *N. farcinica*, by using numerical analysis of phenotypic traits. The unusual phenotypic characteristics that set *N. nova* apart from other members of the "*N. asteroides* complex" were the presence of arylsulfatase activity at 14 days in approximately 70% of the strains and of esterase activity in about 64% of the strains. Tsukamura submitted two reference strains, ATCC 33726 (which was designated the type strain) and ATCC 33727. Because taxonomists were uncertain of the status of these strains, the 1986 edition of *Bergey's Manual of Systematic Bacteriology* listed *N. nova* as a species incertae sedis (120, 121).

A 1988 study of drug susceptibility patterns of isolates within

the *N. asteroides* complex identified a group that was erythromycin and ampicillin susceptible, which was designated the type III drug pattern. This group included the type strain submitted by Tsukamura, *N. nova* (ATCC 33726) (213). In 1990, DNA-DNA hybridization studies established *N. nova* as a valid species, distinct from the *N. asteroides* and *N. farcinica* type strains (ATCC 19247 and ATCC 3318, respectively).

In 1991, Wallace et al., using a combination of biochemical tests and antibiotic susceptibility tests, determined that approximately 20% of the clinical isolates from two reference laboratories in Texas and Minnesota previously categorized as *N. asteroides* had the type III drug pattern and belonged to the species *N. nova* (207). The only commercially available biochemical test useful for the identification of *N. nova* was arylsulfatase activity. A positive arylsulfatase production after 2 weeks, combined with susceptibility to ampicillin and erythromycin, and resistance to amoxicillin-clavulanic acid, were shown to be the best phenotypic means of identification of *N. nova* for the clinical laboratory (207).

In 1995, Steingrube et al. described an RFLP method for identification of 12 species and taxa of *Nocardia*, including *N. nova*, based on a prior study by Lungu and colleagues (131) and using mycobacterial primers developed by Telenti et al. (181, 186). This method involved amplification of a 441-bp region of the 65-kDa heat shock protein (*hsp65* product) and subsequent restriction enzyme digestion with MspI and BsaHI. The technique readily identified most of the commonly encountered *Nocardia* species, with isolates of *N. nova* showing a unique restriction profile of 130-, 110-, and 75-bp bands with MspI and 310-, 70-, and 60-bp bands with BsaHI (181). Two clinical isolates and the *N. nova* type strain (ATCC 33726) exhibited this RFLP pattern. These molecular studies provided further molecular evidence for the species status of *N. nova*.

In 2000, Conville et al., using amplification of a 999-bp region of the 16S rRNA gene and subsequent restriction endonuclease analysis with HinPII and DpnII, showed that an isolate considered to belong to *N. nova* by PRA of the *hsp65* gene exhibited 16S rRNA gene RFLPs different from those obtained with the *N. nova* type strain. Sequence analysis of the 999-bp region of the 16S rRNA gene showed this isolate to have only 98.0% similarity to the *N. nova* type strain, suggesting that some isolates considered to belong to *N. nova* by *hsp65* PRA and antibiotic susceptibility may actually belong to other, unrecognized species (43).

The description of additional species within what had been thought to be the single species *N. nova* began in 2001, when Gürtler and colleagues described a new species that they called *N. veterana* (80). By 16S rRNA gene sequence analysis, *N. veterana* showed 98.6% similarity to the type strain of *N. vac-cinii* (known only to be a plant pathogen) and 98.1% similarity to *N. nova* (80). DNA-DNA hybridization studies showed the type strain of *N. veterana* to be only 33% homologous to *N. nova* (43). Conville and colleagues subsequently showed that three clinical isolates of this species yielded RFLP patterns identical to that of the *N. nova* type strain for the 441-bp amplified portion of the *hsp65* gene (42). Sequence analysis of a 1,359-bp region of the 16S rRNA gene and the 441-bp region of the *hsp65* gene showed the patient isolates to have 100% similarity to the *N. veterana* type strain for both genes (42).

Phenotypic differentiation of the two species is complicated by the finding that *N. veterana* has an antimicrobial susceptibility pattern indistinguishable from that of *N. nova* (42, 161). This includes higher MICs to amoxicillin-clavulanic acid than to ampicillin due to a β -lactamase detectable only after induction with clavulanic acid, a phenomenon originally described by Wallace et al. and also seen in *N. nova* (207). Using phenotypic characteristics alone, isolates of *N. veterana* are not reliably differentiated from *N. nova* (161).

The second strain of *N. nova* submitted by Tsukamura in 1982 (ATCC 33727) was found to have a 16S rRNA gene sequence with 100% similarity to that of *N. veterana* (39, 157), further demonstrating the difficulty in phenotypically separating these two species (39).

In 2001, Hamid et al. described a second new species, *N. africana*, which most closely resembled *N. vaccinii* by 16S rRNA gene sequencing (82). In that study, *N. africana* was found to differ phenotypically from the type strain of *N. nova* in that *N. africana* hydrolyzed only casein and grew at 45°C (it is important to recognize that these data are based on small numbers of isolates and may change with further testing of additional isolates) (82). Hamid and colleagues also report that isolates of *N. africana* are susceptible to amoxicillin, ampicillin, doxycycline, and erythromycin but resistant to gentamicin, kanamycin, and sulfamethoxazole. Amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, amikacin, and clarithromycin were not tested (82). *N. africana* was subsequently shown to exhibit *hsp65* PRA patterns identical to those obtained with *N. nova* and *N. veterana*. Sequence analysis of the 16S rRNA gene showed *N. africana* to be 98.4 and 99.0% similar to the type strains of *N. nova* and *N. veterana*, respectively (42).

In 2004, Conville et al. described a third new species belonging to the *N. nova* complex, *N. kruczakiae*. Like *N. africana*, this species is indistinguishable from *N. nova* and *N. veterana* by *hsp65* PRA and antimicrobial susceptibility testing and is identical to *N. africana* and *N. veterana* by 16S rRNA gene PRA. *N. kruczakiae* shows very high 16S rRNA gene similarity to *N. veterana* (99.8%) and is 99.3 and 98.1% similar to *N. africana* and *N. nova*, respectively. DNA-DNA hybridization studies show it to be a unique species (42).

Conville and colleagues concluded that the optimal phenotypic tests for differentiation among *N. africana*, *N. kruczakiae*, *N. nova*, and *N. veterana* are arylsulfatase production at 14 days, esculin hydrolysis, casein hydrolysis, and acid production from D-galactose, glycerol, raffinose, salicin, and trehalose (41). However, few isolates of each species (with identification established by 16S rRNA gene sequencing) have been analyzed using these phenotypic criteria alone, and therefore identification of these species by phenotypic methods should be considered tentative, with isolates reported as belonging to the *N. nova* complex. Definitive species identification within the complex currently requires molecular testing.

In a recent study by Wilson and colleagues (R. W. Wilson, P. S. Conville, L. Mann, B. A. Brown-Elliott, C. Crist, F. G. Witebsky, and R. J. Wallace, Jr., Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. C-75, 2004), 92 (later expanded to 101) clinical isolates identified as *N. nova* based on *hsp65* PRA results and antimicrobial susceptibility patterns (R. W. Wilson, personal communication, 2004) were subjected to PRA of the 16S rRNA gene. Sixty-four percent of the isolates were deter-

mined to be *N. nova*; of these, 40% showed a variant 16S rRNA PRA pattern that has subsequently been shown to be that of *N. nova* by DNA-DNA hybridization (45). The remainder of the isolates belonged to either *N. kruczakiae*, *N. veterana*, or multiple yet-to-be described taxa. This study demonstrates the heterogeneity of clinical isolates considered to belong to *N. nova* and also demonstrates the need for further DNA-DNA hybridization studies for further characterization of all the species that may be involved (Wilson et al., 104th Gen. Meet. Am. Soc. Microbiol.).

Other studies also suggest that additional species may well be present in isolates identified phenotypically as *N. nova* complex. McNabb and colleagues observed a number of different cell wall fatty acid profiles in a previous study of *N. nova* isolates (135). Patel and coworkers (157) studied nine isolates originally identified as *N. nova* in the 1988 study by Wallace et al. (213). By *hsp65* RFLP, these isolates showed identical patterns with the four restriction enzymes (BstEII, MspI, HinfI, and BsaHI) (181). However, 16S rRNA gene sequencing (the first 500 bp) of the nine isolates produced five unique sequences. The sequences from four clinical isolates were identical to that from the type strain of *N. nova* by 5 bp and were identical to that of ATCC 33727 (known to be identical to *N. veterana*). The sequences of the remaining three clinical isolates differed from that of the *N. nova* type strain by 2, 3, and 5 bases, respectively. Thus, the isolates that were phenotypically different only by their fatty acid profiles (135) differed by partial 16S rRNA gene sequencing from the *N. nova* type strain by 0 to 5 bases. This small study further emphasized the molecular heterogeneity of isolates clinically identified as *N. nova* (157).

Thus, while identification using both phenotypic properties and molecular methods is optimal for the identification of these species, laboratories without molecular capabilities should consider a preliminary designation of "*N. nova* complex," which would include the related species *N. africana*, *N. kruczakiae*, *N. nova*, and *N. veterana* as well as likely several additional species yet to be named (41, 42, 44, 157).

***Nocardia transvalensis* Complex (Includes *N. asteroides* Type IV Susceptibility Pattern)**

The first description of *N. transvalensis* was in 1927, when this species was reported as a cause of mycetoma in an African patient (160). Until the late 1990s, however, only a few isolates of this species had been described in any detail. In 1978 Gordon and colleagues had shown that five isolates of *N. transvalensis* had a distinctive pattern of phenotypic traits. All five of the isolates tested decomposed hypoxanthine, tyrosine, and urea and were negative for casein decomposition. Two of the five isolates decomposed xanthine and adenine. Additionally, all of the isolates hydrolyzed esculin and starch; produced acid from adonitol, erythritol, glucose, and glycerol; and utilized citrate. Four of the five isolates produced acid from mannitol, sorbitol, and trehalose (78).

In 1992, McNeil et al. reported 15 clinical isolates that showed variability in a number of phenotypic characteristics compared to the *N. transvalensis* type strain but were unique in their high degree of antibiotic resistance (137).

In 1997, Wilson et al. characterized 56 clinical isolates of *Nocardia* which they classified as belonging to an "*N. transvalensis* complex" based on the similarities of their antimicro-

bial susceptibility patterns and biochemical characteristics. These isolates were found to belong to four separate groups within the *N. transvalensis* complex. The first group most closely resembled the *N. transvalensis* type strain (223).

The second group of isolates included isolates of *N. asteroides* complex having the type IV antibiotic susceptibility pattern originally noted in the 1988 description of drug patterns in *N. asteroides* (213) and represents the most commonly isolated organism in this complex. The isolates of both type IV *N. asteroides* and *N. transvalensis* were generally resistant to all aminoglycosides, including amikacin, a highly unique pattern among *Nocardia* spp. (223). The 31 isolates of *N. asteroides* type IV were distinguished from *N. transvalensis* by a few phenotypic characteristics (223). The first two taxa of *N. transvalensis* and *N. asteroides* complex type IV exhibit several common biochemical characteristics, including utilization of citrate, D-galactose, and D-trehalose; hydrolysis of hypoxanthine; and similar mycolic acid ester high-performance liquid chromatography (HPLC) patterns (223).

The last groups within the *N. transvalensis* complex were identified and designated *N. transvalensis* "new taxon 1" and "new taxon 2." Eighty-seven percent of these isolates were shown to be susceptible to amoxicillin-clavulanic acid, did not utilize i-myo-inositol, and were separated from each other on the basis of differences in utilization of D-glucitol and D-mannitol and variable hydrolysis of tyrosine (223).

By PCR restriction enzyme analysis of the 441-bp fragment of the *hsp65* gene, isolates belonging to the four groups of the *N. transvalensis* complex were separable, although those of *N. transvalensis sensu stricto* and *N. asteroides* complex type IV were not clearly differentiated from each other (181, 183). Results of PCR-RFLP analysis with HxoI and HinfI are very similar for these two taxa, while analysis with HaeIII results in the complete separation of *N. asteroides* type IV and both new taxons within the *N. transvalensis* group.

McNabb et al. analyzed the fatty acid profiles of 13 *N. transvalensis* isolates and found that none of the clinical isolates matched ATCC 6865^T and, with only two exceptions, they were so dissimilar that they were unable to be grouped (135).

Recently, Patel and colleagues showed that by sequence analysis of the initial 500 bp of the 16S rRNA gene, isolates belonging to each of the subgroups within the complex were more closely related to each other than they were to other members of the complex (157). The four groups within the *N. transvalensis* complex showed from 98.0% to 99.2% similarity to *N. transvalensis* ATCC 6865^T, a difference of between 4 and 10 bp. The only group with 100% sequence identity was the *N. asteroides* type IV drug group (157).

Based upon these findings, the proposed designation for these combined four taxa is *N. transvalensis* complex, pending further genetic studies (157, 223). Although not all isolates may meet the criteria of in vitro resistance to amikacin, an operational definition should include resistance to amikacin, a characteristic unique to this group. Examples of the type IV drug susceptibility pattern (ATCC 49872 and ATCC 49873) and new taxon 1 (ATCC 700034 and ATCC 700035) have been deposited in the American Type Culture Collection (ATCC), Manassas, Va.

***Nocardia farcinica* (Formerly *N. asteroides* Type V Drug Susceptibility Pattern)**

In their study of antibiotic susceptibility patterns of isolates belonging to the *N. asteroides* complex, Wallace et al. designated a group of isolates with a unique pattern that they designated drug pattern type V (213). They subsequently showed that the type strain of *N. farcinica* belonged to this group and commented that a strain marketed as a Preceptrol ATCC reference strain of *N. asteroides* (ATCC 3308) in fact belonged to the species *N. farcinica* (214). By using a battery of biochemical tests, including growth at 45°C, utilization of acetamide as a nitrogen and carbon source, acid production from rhamnose, and resistance to tobramycin and cefamandole, this strain was shown to be typical of *N. farcinica* (214). This was later confirmed by a number of molecular studies (157, 181, 183).

Chemotaxonomic analysis of lipids, 16S rRNA gene sequencing, and DNA hybridization studies (146) have established *N. farcinica* as a distinct species (34). By 16S rRNA gene sequencing, most clinical isolates show 100% homology with the ATCC type strain (157). Steingrube and colleagues showed that *N. farcinica* could be differentiated from other *Nocardia* species by using PRA of the *hsp65* gene (181).

Isolates of *N. farcinica* have a specific drug pattern characterized by resistance to most beta-lactam antibiotics, including cefamandole (100%), cefotaxime (100%), and ceftriaxone (80%). The isolates are also resistant to aminoglycosides, including tobramycin (>90%), kanamycin (100%), and gentamicin (100%), but are susceptible to amikacin (100%). Of the 40 clinical isolates reported by Wallace et al. (214), 100% were also susceptible to sulfamethoxazole, 82% to imipenem, 88% to ciprofloxacin, and only 5% and 3% to ampicillin and erythromycin, respectively (214).

A study by McNabb et al. of the fatty acid compositions of 73 isolates of *N. farcinica* revealed that this species formed a homogeneous group containing a major percentage of straight-chain saturated or monounsaturated types of fatty acids (135).

In 2004, Ishikawa et al. determined the genomic sequence of a clinical strain of *N. farcinica*. The circular chromosome was found to contain over 6 million base pairs; two plasmids and multiple candidate genes for virulence and intrinsic multidrug resistance were also identified (86).

***Nocardia asteroides* (Type VI Drug Susceptibility Pattern)**

N. asteroides drug pattern type VI (213) is the most commonly isolated drug pattern type of the "*N. asteroides* complex" in the southern United States. This organism is difficult to identify phenotypically using commercially available biochemicals. It is most easily recognized by its susceptibility to cefamandole, cefotaxime, ceftriaxone, amikacin, and imipenem and resistance to ciprofloxacin, ampicillin, amoxicillin-clavulanic acid, erythromycin (which presumably applies to clarithromycin), and ciprofloxacin (44, 167, 213, 214). Molecular methodologies, including *hsp65* gene PRA, 16S rRNA gene PRA, and 16S rRNA gene sequencing, have allowed more accurate assignment of clinical isolates to this group. In 2001, Yassin et al. described *N. cyriacigeorgica* from bronchial secretions of a patient with chronic bronchitis (229). 16S rRNA gene sequence analysis of 1,377 bp showed this organism to

have a high degree of sequence similarity to other *Nocardia* species but to be distinct from *N. asteroides*, *N. abscessus*, and *N. paucivorans* by DNA-DNA hybridization. In 2003, Roth et al. showed the 16S rRNA gene sequence of the type strain of *N. cyriacigeorgica* to be identical to that of ATCC 14759, which was selected by Wallace as the reference strain of *N. asteroides* drug pattern type VI (167).

Recently, Patel et al. analyzed five isolates belonging to the type VI drug susceptibility pattern (157). All isolates exhibited similar susceptibility patterns, the same *hsp65* PRA patterns, and similar biochemical results, except for variable results for citrate, rhamnose, and acetamide utilization. All five isolates grew well at 45°C at 3 days. The five isolates had identical partial (500-bp) 16S rRNA gene sequences. This sequence showed 100% sequence similarity to the recently described *N. cyriacigeorgica* (229). Subsequent testing revealed the biochemical profile, *hsp65* PRA pattern, and drug susceptibility pattern of *N. cyriacigeorgica* to be identical to those of the isolates of *N. asteroides* drug pattern type VI. DNA-DNA hybridization studies are still needed to confirm the relationship between *N. cyriacigeorgica* and *N. asteroides* drug pattern type VI.

Nocardia brasiliensis and *Nocardia pseudobrasiliensis*

The species now known as *N. brasiliensis* was originally described by Lindenberg in 1909 as *Discomyces brasiliensis* (125). Other synonyms in the early 20th century literature include *Streptothrix brasiliensis*, *Oospora brasiliensis*, *Actinomyces brasiliensis*, and *Actinomyces violaceus* subsp. *brasiliensis* (121).

N. brasiliensis differs from all other recognized *Nocardia* species in that it has been most often associated with a primary cutaneous infection. These infections usually occur in normal hosts (61).

In the clinical laboratory, isolates of *N. brasiliensis* have traditionally been identified using biochemical methods and cell wall composition (206). *N. brasiliensis* typically decomposes casein and tyrosine and is variable in its decomposition of hypoxanthine. In 1995, Wallace et al. showed that some isolates initially identified as *N. brasiliensis* were phenotypically different from *N. brasiliensis* in their positive hydrolysis of adenine, negative nitrate reduction, and susceptibility patterns. In addition, these isolates were shown to be more often associated with invasive disease in immunocompromised hosts rather than primary cutaneous disease. PRA of the *hsp65* genes of these variant isolates showed RFLPs distinct from those seen for *N. brasiliensis*, suggesting a molecular distinction from *N. brasiliensis* (206). Subsequent 16S rRNA gene sequence analysis and DNA-DNA hybridization showed these isolates to belong to a distinct species, *N. pseudobrasiliensis* (168, 206).

Major differences in susceptibility patterns were also seen when comparing isolates of *N. brasiliensis* and isolates of the new taxon. Because isolates of *N. brasiliensis* are typically resistant to ciprofloxacin and clarithromycin, unlike those of *N. pseudobrasiliensis*, susceptibility testing of these two agents has been suggested as a screening method for the differentiation of isolates of *N. brasiliensis* from *N. pseudobrasiliensis* (206). Additionally, in contrast to the results obtained for *N. pseudobrasiliensis*, the majority of isolates of *N. brasiliensis* are susceptible to amoxicillin-clavulanic acid, trimethoprim-sulfa-

methoxazole (TMP-SMX) (by disk diffusion), and minocycline, in contrast to those of the new taxon (206) (Table 3).

The two taxa are also distinguishable by β -lactamase isoelectric focusing patterns. The majority of the *N. brasiliensis* isolates exhibit a common double-band β -lactamase pattern with major band pIs at 5.00 and 5.05, whereas 25% of the new taxon showed lower pIs of 4.73 and 4.91 (206, 208). This β -lactamase activity probably confers resistance to multiple β -lactam agents, including cephalosporins, benzylpenicillin, cefoxitin, and imipenem (208).

Furthermore, chemotaxonomically isolates of *N. pseudobrasiliensis* exhibited a unique HPLC pattern containing two early peaks of mycolic acids not seen in *N. brasiliensis* sensu stricto (168). Two strains of *N. pseudobrasiliensis*, ATCC 51511 and ATCC 51512^T, were deposited in the American Type Culture Collection.

Nocardia otitidiscaviarum Complex (Formerly *N. caviae*)

N. otitidiscaviarum was first recognized by Snijders in 1924 from a Sumatran cavy or guinea pig with ear disease (179). “Caviarum” refers to the generic name of the cavy or guinea pig, and “otitis” refers to inflammation of the ear. In 1935, Erikson renamed the organism *Actinomyces caviae* (55). Subsequently, Gordon and Mihm placed the organism in the genus *Nocardia* and proposed the name “*Nocardia caviae*” (77). They also stated that the name *N. otitidiscaviarum* was not proper according to the 1958 International Code of Nomenclature of Bacteria and Viruses (77). However, *Bergey's Manual of Systematic Bacteriology* currently uses the name “*N. otitidiscaviarum*” (121), and this name is now routinely used in the clinical laboratory to identify members of this complex (56).

N. otitidiscaviarum has been recovered from soil and has also been described as an opportunistic pathogen for humans and animals (72, 117). In humans, *N. otitidiscaviarum* is known to be a cause of primary cutaneous, lymphocutaneous, and pulmonary infections in both immunocompetent and immunocompromised patients (30, 36, 50, 203). Infections due to *N. otitidiscaviarum* appear to be rare compared to those caused by other species of *Nocardia*, and there were no published reports of human infection until the mid 1960s (36). Beaman et al. reported that only 10 (2.9%) of 347 cases of infections due to *Nocardia* in the United States were identified as due to *N. otitidiscaviarum* (9). It has also been postulated that *N. otitidiscaviarum* is less pathogenic than other species of *Nocardia*, but this has not been proven and is controversial because of the ability of the organism to cause infection in laboratory animals (36, 81).

Biochemically, *N. otitidiscaviarum* is distinct from other *Nocardia* species in its ability to hydrolyze both hypoxanthine and xanthine. However, the lack of commercial sources of these media may make phenotypic identification difficult. See Table 4 for a summary of biochemical characteristics of this species. Molecular testing, including 16S rRNA gene sequence analysis, may be necessary for confirmation of the species identification.

Recently 10 clinical isolates of *N. otitidiscaviarum* complex were studied by Patel et al., using *hsp65* PRA and 16S rRNA gene sequence analysis of the first 500 bp (157). Although their antimicrobial susceptibilities and biochemical profiles were similar, by *hsp65* PRA with three restriction enzymes (BstEII,

TABLE 4. Major phenotypic characteristics of the nocardiae^a

Species or strain	Growth at 45°C	Production of:			Hydrolysis of:						Utilization of:			
		Arylsulfatase (14 days)	Nitrate reductase	Urease	Adenine	Casein	Esculin	Hypoxanthine	Tyrosine	Xanthine	Acetamide ^b	Citrate ^c	L-Rhamnose ^c	D-Sorbitol ^c
<i>N. abscessus</i>	-	-	+	+	-	-	-	-	-	-	-	+	-	-
<i>N. africana</i>	+	+	-	-	-	+	-	-	-	-	NA	-	-	-
<i>N. alba</i>	-	NA	+	NA	-	-	NA	NA	NA	NA	NA	-	+	-
<i>N. anaemiae</i>	-	NA	NA	+	-	-	NA	+	-	-	NA	+	-	+
<i>N. aobensis</i>	-	NA	NA	+	-	-	NA	-	-	-	NA	-	-	-
<i>N. araoensis</i>	+	-	-	+	NA	-	-	NA	NA	-	-	+	-	NA
<i>N. arthritis</i>	-	-	+	+	-	-	W	-	-	-	NA	+	-	-
<i>N. asiatica</i>	-	-	+	V	-	-	-	-	-	-	NA	+	+	-
<i>N. asteroides</i> ATCC 19247 ^T	-	-	NA	+	-	-	-	-	-	-	-	-	-	-
<i>N. beijingensis</i>	-	NA	NA	+	-	-	-	-	-	+	NA	+	+	+
<i>N. brasiliensis</i>	-	-	+	+	-	+	+	+	+	-	-	+	-	-
<i>N. brevicatena</i>	-	-	-	-	-	-	+	-	-	-	-	-	-	-
<i>N. caishijiensis</i>	-	-	+	-	-	-	+	-	-	-	NA	-	-	-
<i>N. carnea</i>	-	NA	+	-	-	-	+	-	-	-	NA	-	-	+
<i>N. cerradoensis</i>	-	NA	+	+	-	-	+	-	-	-	-	-	+	-
<i>N. coeliaca</i>	-	NA	NA	NA	NA	-	NA	-	+	-	-	-	+	-
<i>N. crassostreae</i>	-	NA	NA	V ^d	NA	-	-	-	-	-	NA	-	-	-
<i>N. cummidelens</i>	-	NA	+	+	-	-	-	-	-	-	NA	-	-	-
<i>N. cyriacigeorgica</i>	+	NA	+	-	-	-	-	-	-	-	+	-	-	-
<i>N. farcinica</i>	+	-	-	+	-	-	+	-	-	-	+	-	+	-
<i>N. flavorosea</i>	+	NA	-	-	-	-	-	-	-	-	NA	+	-	-
<i>N. fluminea</i>	-	NA	+	-	-	-	+	-	+	-	-	+	+	-
<i>N. higoensis</i>	+	NA	NA	+	NA	NA	NA	NA	NA	-	NA	-	-	-
<i>N. ignorata</i>	-	-	+	+	-	-	+	-	-	-	NA	-	-	-
<i>N. inohanensis</i>	-	NA	NA	+	-	-	NA	+	-	-	NA	+	-	-
<i>N. kruczakiae</i>	+ ^e	+	NA	NA	-	-	+	-	-	-	-	+	-	-
<i>N. mexicana</i>	-	-	NA	+	+	-	NA	+	-/W	-	NA	NA	+	+
<i>N. neocaledoniensis</i>	+	NA	+	+	NA	-	+	-	+	-	NA	+	NA	NA
<i>N. niigatensis</i>	-	NA	NA	V	-	-	NA	W	-	-	NA	-	-	-
<i>N. nova</i>	-	+	+	+	-	-	-	-	-	-	-	-	-	-
<i>N. otitidiscaviarum</i>	V	NA	+	+	-	-	+	+	-	+	-	-	-	-
<i>N. paucivorans</i>	+	NA	-	+	-	-	+	-	-	-	-	-	-	-
<i>N. pigrifrangens</i>	NA	NA	+	+	-	-	-	-	-	-	-	-	-	-
<i>N. pneumoniae</i>	-	W	+	-	NA	+	+	NA	NA	-	+	+	NA	NA
<i>N. pseudobrasiliensis</i>	-	-	-	+	+	+	+	+	+	-	NA	+	-	-
<i>N. pseudovaccinii</i>	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	-	-	-	-
<i>N. puris</i>	-	NA	+	+	-	-	+	-	-	-	-	+	-	+
<i>N. salmonicida</i>	-	NA	+	+	-	-	+	-	+	+	-	+	+	+
<i>N. seriola</i>	-	NA	+	-	-	-	+	-	-	-	NA	-	-	-
<i>N. shimofusensis</i>	-	NA	NA	+	NA	NA	NA	NA	NA	-	NA	-	-	-
<i>N. sienata</i>	-	NA	NA	-	-	NA	-	-	-	-	NA	-	-	-
<i>N. soli</i>	-	-	+	+	-	-	+	-	-	-	NA	+	+	-
<i>N. takedensis</i>	-	NA	NA	NA	NA	-	+	-	-	-	NA	NA	-	-
<i>N. tenerifensis</i>	NA	NA	NA	+	-	+	+	+	+	-	NA	+	-	-
<i>N. testaceae</i>	+	NA	NA	-	-	-	-	-	-	-	NA	-	+	-
<i>N. thailandica</i>	-	NA	NA	+	-	-	NA	-	-	-	NA	-	-	-
<i>N. transvalensis</i> complex	V	-	+	+	-	-	+	+	-	-	-	+	-	V
<i>N. uniformis</i>	-	NA	+	+	-	-	-	+	+	-	NA	-	-	-
<i>N. vaccinii</i>	-	-	+	+	-	-	-	+	-	-	NA	+	+ ^f	-
<i>N. vermiculata</i>	-	NA	NA	-	-	-	NA	-	-	-	NA	-	-	-
<i>N. veterana</i>	+	-	-	+	-	-	-	-	-	-	-	-	-	-
<i>N. vinacea</i>	-	NA	+	+	-	-	d	+	-	-	NA	d	-	+
<i>N. xishanensis</i>	-	NA	+	NA	-	-	-	-	-	-	NA	-	+	+
<i>N. yamanashiensis</i>	-	NA	NA	+	-	-	-	+	-	-	NA	+	-	-

^a Symbols and abbreviations: -, negative; +, positive; NA, not available; V, variable; d, doubtful; W, weak reaction. Composite data are from all references from Tables 1 and 2 and also from references 42, 72, 167, 168 and 223.

^b Utilization as sole source of carbon and nitrogen.

^c Utilization as sole source of carbon.

^d Results vary according to method.

^e Optimal growth.

^f Results differ in separate publications (42, 80).

MspI, and HinfI), three different RFLP patterns were noted. A fourth pattern was seen when an additional restriction enzyme (BsaHI) was employed. Thirty-six percent of isolates failed to produce an amplicon after PCR with the standard primers. By 16S rRNA partial gene sequencing, three clinical isolates had a sequence identical to that of the type strain, ATCC 14629, and these were most closely related to the sequences found in the MicroSeq and GenBank databases. The remaining six isolates had sequences similar to each other but different from that of the *N. otitidiscaviarum* type strain ATCC 14629, suggesting that *N. otitidiscaviarum* may represent a heterogeneous complex of organisms (157).

The degree of heterogeneity among isolates identified as *N. otitidiscaviarum* complex was also emphasized in a study of fatty acid profiles among 20 strains of this organism (135). The strains were separated into three groups, with reference strains ATCC 14629^T, ATCC 14630, and ATCC 27941 representative of the three groups that the authors designated A, B, and C, respectively (135). Because of the chemotaxonomic and molecular heterogeneity, Patel et al. (157) proposed that clinical isolates of *N. otitidiscaviarum* be called members of the *N. otitidiscaviarum* complex (as recommended for *N. nova*, *N. transvalensis*, and *N. asteroides*) until further taxonomic clarification is available (157).

Isolates of *N. otitidiscaviarum* complex are usually resistant to beta-lactams, including most broad-spectrum cephalosporins, ampicillin, amoxicillin-clavulanic acid, and imipenem, but are usually susceptible to amikacin, the fluoroquinolones, and sulfonamides (Table 3).

CLINICAL DISEASE ASSOCIATED WITH *NOCARDIA*

Clinical Significance of the Nocardiae in Culture

Because the nocardiae are ubiquitous in the environment, the isolation of these organisms may be indicative of laboratory contamination or, especially in respiratory samples, may indicate colonization and not invasive infection. Furthermore, the nocardiae may exist as saprophytes on the skin and in the upper respiratory tract.

Criteria helpful for ascertaining the clinical significance of a culture positive for a *Nocardia* sp. include signs and symptoms of the patient that correlate to infection with *Nocardia* spp. The clinical setting is also important (e.g., patient on corticosteroids, renal transplant, etc.).

In the laboratory, visualization of the typical branching morphology of the organism surrounded by acute inflammatory cells on a direct Gram-stained smear is the first and one of the most important findings. Pure or large quantities of the organisms in culture and isolation of the organisms from normally sterile body sites are also factors in determining the clinical relevance of the presence of the organism in culture. Isolation of a *Nocardia* sp. from a single broth culture does not necessarily imply infection with the organism. Indeed, isolation of the *Nocardia* sp. from multiple clinical samples helps to establish the organism as clinically significant. "Colonization" without disease of the respiratory tract is seen only in patients with bronchiectasis, including patients with cystic fibrosis and older women with *Mycobacterium avium* complex. Given these comments, environmental contamination of a clinical specimen is

rare. "Colonization" in the setting of bronchiectasis without "disease" does occur, however.

Pulmonary Disease

Most *Nocardia* infections in the United States are believed to be acquired by inhalation of airborne spores or mycelial fragments from environmental sources (172, 173). The most common manifestation of nocardial disease is pulmonary nocardiosis, occurring most frequently in immunocompromised patients (127, 136, 139, 140). Some patients with pulmonary nocardiosis also have underlying chronic lung disease such as chronic obstructive pulmonary disease (emphysema or chronic bronchitis), asthma, chronic sarcoidosis, or bronchiectasis and, as Saubolle and Sussland note, often (with the exception of bronchiectasis) have been treated with long-term, high-dose corticosteroid therapy (173).

Patients with drug (usually corticosteroid)-induced systemic immunosuppression (including hematologic malignancies, organ transplants, and systemic lupus erythematosus) and patients with non-drug-induced immunosuppression (including chronic granulomatous disease [33, 51, 90, 128, 147], chronic alcoholism, diabetes mellitus, and human immunodeficiency virus [HIV] infection) are also more susceptible to pulmonary infections with *Nocardia* (138).

Pulmonary nocardiosis may present as a subacute or indolent disease, with symptoms present from one to several weeks. Cough is usually predominant, and patients may produce a thick purulent sputum. Fever, weight loss, and malaise similar to the symptoms of nontuberculous mycobacterial disease may also be common.

Infiltrates are usually seen on X ray and may be accompanied by single or multiple nodules that often appear smooth and round. Cavitation of an infiltrate or nodule is common, and in some series empyema is present in one-third of the cases (61). The differential diagnosis usually includes malignancy, pulmonary tuberculosis, *Rhodococcus equi* (in HIV-infected patients), or fungal pneumonia, especially *Aspergillus*.

Extrapulmonary Disease

One-half of all cases of pulmonary nocardiosis also involve infections in areas outside the lungs, and approximately 20% of patients with disseminated disease present solely with extrapulmonary disease, which usually has spread hematogenously from an asymptomatic or healed pulmonary site (61). Extrapulmonary local extension may also occur from the lungs, resulting in purulent pericarditis, mediastinitis, and/or the superior vena cava syndrome (1, 154, 222). *Nocardia* sepsis (bacteremia) is unusual but has been reported to result from either pulmonary or extrapulmonary sites of initial infection (17, 114, 115, 128, 164).

Other common sites of nocardial dissemination include skin, subcutaneous tissues, and the central nervous system (53, 61, 106, 110, 188). Unusual extrapulmonary manifestations include *N. farcinica* infection of sternotomy sites following open heart surgery (219), *N. asteroides* complex peritonitis following continuous ambulatory peritoneal dialysis in a patient with systemic lupus erythematosus and chronic renal failure (128), and *N. asteroides* complex endophthalmitis in transplant recipients receiving corticosteroid therapy (113).

Immunocompromised hosts, including patients with AIDS (199, 204), malignancies (61, 187), diabetes, (33, 200, 204), rheumatological disease, or organ transplants or those patients undergoing immunosuppressive therapies utilizing corticosteroids, chemotherapy, and antirejection regimens (20, 28, 58, 66, 123, 147), are more commonly affected.

Many cases of disseminated nocardiosis are associated with CNS disease. Patients with CNS nocardiosis usually have one or more brain abscesses (110). They often present with signs of increased intracranial pressure, including headache, nausea, vomiting, confusion, and depressed consciousness. Seizures are not an uncommon presentation for *Nocardia* brain abscess. Symptoms tend to be more indolent than with bacterial brain abscess. Murray and colleagues described an immunocompetent patient with multiple cerebral lesions due to *Nocardia* that were initially thought to be tumor metastasis (151). Other cases of cerebral nocardiosis in patients with AIDS have also been reported (3, 106).

Meningitis is a less common manifestation of CNS nocardiosis (61, 221). Eisenblätter and colleagues described a case of *N. paucivorans* meningitis in a patient with underlying chronic lung disease (53).

Primary Cutaneous Disease

Unlike pulmonary and disseminated nocardial disease, which primarily affect immunocompromised patients, primary cutaneous nocardiosis is usually an infection in immunocompetent hosts. Although primary cutaneous infections may be caused by any species of *Nocardia*, *N. brasiliensis* is the species isolated from the majority (approximately 80%) of cases of primary cutaneous or subcutaneous nocardiosis (134, 171).

Primary cutaneous infections usually presents as lymphocutaneous infection, superficial cellulitis, or localized abscess and usually involve the face in children or the lower extremities in adults (136, 171).

Mycetoma is a late-stage infection and is characterized by a chronic, localized, slowly progressive, and often painless subcutaneous and bone disease usually involving the foot. Most lesions have been present for six months or longer before diagnosis. Walking barefoot may be responsible for the acquisition of many of these infections, since this practice exposes the feet to repeated trauma and inoculation of the organism from soil. Tumefaction, subcutaneous nodules, destructive granulomata, and formation of intermittent fistulas, with production of pus, and granules of various sizes and colors are characteristic features of these infections (61, 136). Mycetoma is rare in developed countries, as patients generally come to medical attention before the disease becomes this far advanced.

Lymphocutaneous infection is typically marked by the presence of a primary pyodermatous lesion (ulceration with purulence), often with areas of chronic drainage and crusting. The organism invades more deeply to involve the lymphatic system and progresses to the formation of lymphatic abscesses, and thus the name lymphocutaneous syndrome is used (136). This form of nocardiosis is similar in appearance to cutaneous sporotrichosis and hence is also often referred to as "sporotrichoid-type" disease (61).

Superficial cutaneous nocardiosis is the least serious of the

cutaneous infections. This form of nocardiosis usually occurs in an immunocompetent individual 1 to 3 weeks following some type of local trauma with subsequent environmental contamination of the wound. Cellulitis is usually subacute with pain, swelling, erythema, and warmth at the affected site. Although the lesions are usually not draining, cellulitis may rarely disseminate to other areas including bone, muscles, and joints. Fergie and Purcell recently detailed 31 cases of cutaneous *N. brasiliensis* in children from South Texas. None of the patients were immunocompromised as a consequence of chronic underlying disease or drug therapy (60). Minor disease such as this may resolve without specific drug therapy or may go undiagnosed.

Secondary cutaneous involvement as a result of disseminated disease also occurs, and is relatively common (102, 129).

Catheter-Related Infections/Bacteremia

Although nocardiosis can involve nearly every organ by hematogenous dissemination, *Nocardia* bacteremia is rare and bacteremia related to central venous catheters (CVCs) is even more unusual (17, 60, 114, 115). For example, Torres and colleagues evaluated the records of cancer patients with nocardiosis at the University of Texas M.D. Anderson Cancer Center, Houston, between 1988 and 2001 and found only 3 (7%) patients with CVC-related *Nocardia* bacteremia (187).

In a separate study, six detailed cases of CVC-related *Nocardia* bacteremia were reported; two of these were in pediatric patients with Port-A-Cath implantations for administration of chemotherapy. Five out of six of the cases were cured when the catheter was removed and short-term antibiotic therapy was used (59). Only one patient in whom the catheter was not removed, experienced recurrent infection. Two of the patients were cured with the catheter left in situ following antibiotic treatment and cessation of chemotherapy and immunosuppressant therapy (59).

In contrast to non-catheter-related *Nocardia* bacteremia, which has a high mortality rate, CVC-related *Nocardia* bacteremia appears to have a more benign course, and none of the patients in this review series died (59). Further studies need to be done to show if antibiotic treatment is needed in addition to removal of the catheter or whether the catheter could be successfully salvaged through antibiotic treatment (59).

Health Care-Associated Disease

Few studies have addressed the issue of health care-associated acquisition or transmission of the nocardiae. Furthermore, even fewer of the reported presumptive clusters have been investigated using modern molecular technology, and the modes of transmission have not been determined. All of the earlier studies were based on the temporal relationship of contacts between the infected patients and environmental cultures that yielded isolates of *Nocardia* that were consistent by susceptibility pattern with the "source" *Nocardia* (7, 48, 83, 84).

One early study involved three outpatients with underlying lymphoma or leukemia (48), while another cluster occurred in seven patients in a renal transplant unit in a London hospital (84). Dust and air samples inside the transplant unit yielded *Nocardia* isolates with similar susceptibility patterns. A subse-

quent report described six other cases, but environmental samples failed to show the organism (83). Baddour and colleagues reported three cases of nocardiosis in patients who received renal transplants within 10 days of each other, with at least two of them possibly infected by the same strain as determined by comparison of biochemical and susceptibility results for the strains. Multiple environmental samples were negative for *Nocardia* spp. The hypothesis was that one patient, who was an active gardener, may have been colonized with *Nocardia* and served as a source of the isolate (7).

Schaal and Lee described a cluster of infections with *N. farcinica* in patients who underwent cardiac, vascular, or transplantation surgery (175). Nine of the 14 patients had no additional immunocompromising condition, and the source of the *N. farcinica* was not definitely established, but there were indications that some of the infections may have been related to dust contaminating the air system in the operating room (174, 175). Again, however, no molecular strain comparison was performed to conclusively identify the relationship among the strains. Only culture results were used to compare the isolates.

In another retrospective clinical epidemiologic study in the United States, investigation of possible clustering of cases of *Nocardia* among immunocompromised heart transplant patients failed to yield data that would implicate a point source of infection (178).

Exmelin et al. reported the use of randomly amplified polymorphic DNA (RAPD) analysis and rRNA gene restriction patterns (ribotyping) in the investigation of three heart transplant recipients with *N. farcinica* within a 2-month time period in the same care unit (57). Although ribotyping did not differentiate the strains adequately, the RAPD procedure produced sufficiently highly polymorphic patterns to distinguish the strains from four unrelated control strains.

Another study, by Wenger and coworkers, was able to document source and nosocomial transmission epidemiologically by using ribotyping analysis of isolates of *N. farcinica* from five patients who developed sternotomy infections following open heart surgery. Four patient isolates and a hand isolate from an uninfected anesthesiologist yielded identical ribotype patterns (219). The fifth case patient's isolate and multiple isolates from the anesthesiologist's hands and home had a different ribotype pattern.

Blümel et al. recently utilized pulsed-field gel electrophoresis (PFGE) to determine that 18 isolates of *N. farcinica* from postoperative infections in a single German hospital surgical ward belonged to a common endemic genotype. Furthermore, organisms of this genotype were detected in air samples from the area and were found to be the cause of three additional pulmonary infections in an area located in proximity to the surgical ward (15).

Unlike pseudo-outbreaks related to mycobacteria, only a few pseudo-outbreaks involving *Nocardia* have been published to date. The first pseudo-outbreak was associated with a contaminated needle in an automated mycobacterial culture system. The similarity of the pseudo-outbreak strains was confirmed by DNA fingerprinting. Environmental cultures were negative for *Nocardia*, and the end of the pseudo-outbreak was associated with changing the needle sterilizer and prolonging the needle sterilization time of the automated system (158).

Molecular strain typing by PFGE and RAPD analysis was also used to investigate a cluster of four isolates identified as *N. asteroides* that were associated with the same type of automatic culture system as previously reported. In this second pseudo-outbreak, an instrument motor drive misalignment resulted in inadequate needle sterilization and cross-contamination over a 6-week period. Environmental cultures from the needle, needle heater, and tubing of the instrument yielded isolates which were found by both methods of molecular typing to be related to a patient isolate that had been processed in the instrument. Both PFGE and RAPD-PCR also clearly differentiated the pseudo-outbreak strain from epidemiologically unrelated clinical isolates of *Nocardia* species (130).

Other Clinical Manifestations of Nocardiosis

Ocular nocardiosis is an uncommonly reported clinical entity. Infection may develop after minor trauma to the eye in healthy individuals, following ocular surgery such as cataract extraction, or following hematogenous dissemination in immunocompromised patients (38, 108, 113). Ocular pathology of nocardiosis includes uveitis, exudative choroiditis, retinal abscess, retinal detachment, keratitis, and iritis. Nocardial endophthalmitis is associated with a high mortality, and survivors have invariably had total blindness in the involved eye (113, 155).

Peritonitis is a common problem in patients undergoing continuous ambulatory peritoneal dialysis. *Nocardia* peritonitis is not symptomatically different from other causes of peritonitis, and there have been few reported cases in the literature. It appears there are no obvious predisposing factors to the development of *Nocardia* peritonitis. Abscess formation is rare, but once diagnosed, surgical treatment and prolonged antimicrobial therapy are indicated (52, 128).

Nocardia synovitis is rare. The majority of the previously reported cases have been in immunocompromised patients and have been an expression of disseminated *Nocardia* (220).

Treatment of *Nocardia* Infections

Since the 1940s the sulfonamides have been the treatment of choice for the treatment of nocardiosis (10, 67, 209). Prior to the introduction of these agents, the fatality rate of pulmonary nocardiosis was nearly 100% (176).

Despite the utility of the sulfonamides in the treatment of the majority of clinical disease due to the nocardiae, not all patients did well. With only sulfonamide treatment, the mortality rate for patients with CNS *Nocardia* infections including brain abscess was almost 50%, with most patients requiring surgical drainage. In addition, patients with non-CNS overwhelming or disseminated disease had a high mortality rate when treated with sulfonamides alone (209, 211).

The combination of sulfamethoxazole with trimethoprim is often used for treatment of nocardiosis. It is generally thought the activity of the TMP-SMX is related only to the sulfonamide portion of the drug (211). Therefore, it is not clear that the TMP-SMX combination provides an advantage over the use of a sulfonamide alone (211). However, in the United States, TMP-SMX is the only commonly available sulfonamide, and thus it is almost always used for therapy of nocardiosis.

Although sulfonamides and TMP-SMX have been the agents of choice in the past, optimal antimicrobial therapy for patients with nocardiosis has yet to be established (139). In a recent review of nocardiosis in patients with AIDS, the majority of these patients were unresponsive to TMP-SMX (127). Given the high mortality of CNS nocardiosis and of overwhelming and/or disseminated disease treated with sulfonamides alone, the poor treatment response of most HIV-infected patients, and the high incidence of adverse events (allergic reactions or severe nausea) occurring with sulfonamide therapy, most clinicians now administer combination drug therapy, at least initially. Thus, it is important to identify alternative antimicrobials and perform in vitro susceptibility testing with these agents to determine their efficacy against isolates of *Nocardia*. The recently approved Clinical and Laboratory Standards Institute (CLSI) (formerly NCCLS) document on susceptibility testing of mycobacteria and other aerobic actinomycetes recommends that all clinically significant isolates of *Nocardia* species be tested for susceptibility to multiple antimicrobial agents (152).

For empirical therapy prior to the availability of susceptibility test results, most clinicians recommend a three-drug regimen consisting of TMP-SMX, amikacin, and either ceftriaxone or imipenem for patients with serious disease, CNS disease, and/or disseminated disease (123, 205). Sulfonamides (or sulfamethoxazole-trimethoprim combination) remain the drug of choice for all forms of *Nocardia*, and the use of amikacin plus a beta-lactam ensures that all isolates (species) will be susceptible to at least one other drug (no species are resistant to both amikacin and a beta-lactam, though some are resistant to one or the other). The recently introduced agent linezolid offers an additional option for amikacin or the beta-lactam or potentially both, given its clinical activity against all species of *Nocardia*. In addition, it is administered orally, while amikacin, ceftriaxone, and imipenem are administered intravenously. However, clinical experience with linezolid is limited (147).

Wallace et al. designed a study to evaluate the effect of treatment with amikacin or sulfonamides on infection caused by *N. asteroides* with the use of murine models. The investigators found that treatment with either amikacin or sulfonamides for 2 to 3 weeks increased the rate of resolution of infection (209). Later, Gombert and colleagues used a mouse model of cerebral nocardiosis to evaluate the relative efficacy of four antimicrobials, i.e., TMP-SMX, amikacin, imipenem, and minocycline (68, 69). Of the four agents tested, amikacin and imipenem were found to be the most effective agents against a single strain of *N. asteroides* complex (which in neither this study nor the previously mentioned study was identified to species using modern taxonomic methods) (68, 69).

Of the tetracyclines, minocycline has the best in vitro activity and has been used for patients who are allergic to sulfonamides (176). In one study, a 400- to 600-mg daily regimen of minocycline was successfully used in the treatment of pulmonary nocardiosis in a patient who failed prior therapy with TMP-SMX (6). However, other patients have not responded as well to minocycline therapy, and the drug is rarely used once susceptibility results are available.

Other antimicrobials, including cephalosporins (cefpirome, cefepime, ceftriaxone, and cefotaxime), have also been studied in vitro against isolates of *Nocardia*. Generally these agents

were more effective in vitro against isolates of the *N. asteroides* type VI drug pattern, *N. abscessus*, *N. nova* complex, and *N. transvalensis* complex than against *N. farcinica*, *N. otitidiscaviarum*, and *N. brasiliensis* (12; B. A. Brown-Elliott, C. J. Crist, S. C. Ward, and R. J. Wallace, Jr., Abstr. 101st Gen. Meet. Am. Soc. Microbiol., abstr. C-374, 2001). The majority of the isolates of *N. farcinica* are resistant to the cephalosporins, including the broad-spectrum cephalosporins (214).

Susceptibilities of *Nocardia* to carbapenems and quinolones have been reported, although not all strains and species are inhibited. Khardori et al. reported an MIC₅₀ of 1 µg/ml for imipenem against 26 isolates of the *N. asteroides* complex (these isolates were not sufficiently characterized to the species level by modern standards and almost certainly contained multiple species with expected different susceptibilities), compared to an MIC₅₀ of ≥64 µg/ml for isolates of *N. brasiliensis* and *N. otitidiscaviarum* (105). However, in comparisons of meropenem and imipenem, meropenem was less active against isolates of *N. farcinica* and *N. nova*, in contrast to having increased activity against other *Nocardia* species (C. J. Crist, B. A. Brown-Elliott, L. B. Mann, and R. J. Wallace, Jr., Abstr. 103rd Gen. Meet. Am. Soc. Microbiol., abstr. U-088, 2003). In a later study, Kageyama et al., showed variable susceptibility to imipenem for clinical isolates of *N. brasiliensis* from Japan (91). In the study by Khardori et al., cefmetazole showed good in vitro activity against isolates of *N. brasiliensis* and the *N. asteroides* complex (in studies by both groups, isolates of "*N. asteroides*" were not identified to species level), in contrast to the activity with *N. otitidiscaviarum*, and the majority of isolates of all three species showed resistance to ciprofloxacin (105). In a later study, the newer 8-methoxy fluoroquinolones, gatifloxacin and moxifloxacin, generally were more active than ciprofloxacin against isolates of nocardiae, especially against isolates of *N. farcinica* and *N. brasiliensis* (Crist, et al., Abstr. 103rd Gen. Meet. Am. Soc. Microbiol.).

Isolates of the *N. asteroides* type VI drug susceptibility pattern are not susceptible to β-lactam and β-lactamase inhibitor combinations such as amoxicillin-clavulanic acid. However, isolates of *N. brasiliensis* are usually susceptible to amoxicillin-clavulanic acid, and it is the preferred oral agent for this species for patients who cannot tolerate a sulfonamide (208, 215). A case of acquired resistance to β-lactam and β-lactamase inhibitor antibiotics was previously described by Steingrube and colleagues (182). Other species with susceptibility to amoxicillin-clavulanic acid include *N. abscessus* and *N. farcinica*, while isolates of the *N. nova* complex are ampicillin susceptible/intermediate but resistant to amoxicillin-clavulanic acid.

Recently, a new class of antibiotics, the oxazolidinones, was introduced. Linezolid, the first agent in this class to be studied against *Nocardia*, showed extraordinary in vitro activity against all of the major clinically significant species of *Nocardia*. The MIC₉₀ for linezolid was ≤4 µg/ml against isolates of *N. asteroides*, *N. farcinica*, *N. nova*, *N. brasiliensis*, *N. pseudobrasiliensis*, the *N. transvalensis* complex, and *N. otitidiscaviarum* (27). Thus, it is the first antimicrobial agent other than the sulfonamides to be active against most or all isolates of all pathogenic species of *Nocardia*. Subsequently this agent was successfully used to treat patients with nocardiosis, including patients who had failed therapy with other agents (147, 201).

LABORATORY EVALUATION OF *NOCARDIA*

Diagnosis/Identification

Microscopic and macroscopic direct examination. Bronchial washings, bronchial lavage fluids, sputum samples, abscesses, wound drainages, tissues, or cerebrospinal fluids are the most commonly received specimens. The initial macroscopic inspection should include examination for the presence or absence of granules, and if granules are present, they should be carefully washed in sterile saline, crushed, and examined microscopically. These granules are most often seen in infections with *N. brasiliensis* but also can be produced by other *Nocardia* species and with *Actinomyces* species (136).

It is important that physicians notify the laboratory when *Nocardia* is suspected in a clinical specimen so that measures can be taken to optimize recognition and recovery of the organism. Microscopic and macroscopic examinations of specimens submitted for culture of *Nocardia* are the first steps in providing a definitive diagnosis. Stainings with modified acid-fast stains, and especially Gram stains, are especially important to provide rapid presumptive diagnosis while awaiting the results of the culture (11, 24, 173).

Although the nocardiae are not always seen on smears of clinical material, optimally smears should be prepared in duplicate, with one slide used for Gram stain and the second slide used for the modified Kinyoun stain. In clinical specimens, nocardiae are seen microscopically as beaded gram-positive, thin, branching, filamentous organisms, usually in a background of purulence with many polymorphonuclear leukocytes. Gram staining is the most sensitive method by which to visualize and recognize nocardiae in clinical samples (173).

With the modified Kinyoun acid-fast stain using weak acid (1% sulfuric acid) for decolorization, the nocardiae are often seen as partially acid-fast filamentous bacilli (showing both acid-fast and non-acid-fast organisms). This acid fastness may be variable when staining colonies of *Nocardia* from cultured material. Additionally, results of the colonial acid-fast smear may be related to the type of medium used and the age of the culture, and filaments may fragment into bacilli or coccoid forms. Because of the difficulties in the preparation and interpretations of the modified acid-fast stain, these smear results should be used judiciously and in conjunction with other laboratory tests and the patient's clinical history (24, 136). Use of the modified acid-fast stain with direct clinical samples is unreliable and should be used only to confirm the acid fastness of organisms detected by the Gram stain (173).

Cultures for nocardiae require a minimum of 48 to 72 h before colonies are evident (70). Colonial morphology should also be observed macroscopically and microscopically for the presence of aerial hyphae. The use of a stereoscope will allow early recognition of the aerial hyphae of *Nocardia* isolates, which will impart a "cotton candy" appearance to the colony surface. McNeil and Brown have described a procedure for demonstrating the microscopic morphology of a nocardial isolate by in situ observation of growth on a slide culture containing a minimal medium such as tap water agar or cornmeal agar without dextrose (136). Slide cultures are incubated at 25°C and examined periodically for 2 to 3 weeks for the appearance of the substrate and aerial mycelium and for the presence of

sporulation. Although they may be subtle, or may be absent in rare cases, the presence of aerial hyphae will differentiate the genus *Nocardia* from related genera, including *Rhodococcus*, *Gordonia*, *Tsukamurella*, *Corynebacterium*, and *Mycobacterium* (24, 136). *Streptomyces* species and other members of the aerobic actinomycetes may also show aerial hyphae, but these genera will not give a positive result with the modified acid-fast smear. On slide culture *Nocardia* will show complex substrate hyphae branching at right angles and usually with secondary branching.

Colonial morphology of the nocardiae is variable, from the smooth, almost bacterium-like appearance of *N. farcinica*, which turns orange with age, to a more chalky white appearance of most other species, depending on the presence of aerial hyphae. Isolates of *N. brasiliensis* typically have a yellowish coloration, and isolates of *N. asteroides* type VI frequently produce a brown diffusible pigment that is rarely seen with other *Nocardia* species. The appearance may also differ depending upon the medium or incubation temperature. Nocardiae grow readily on most nonselective laboratory media, but colonies may take from 2 to 14 days to appear (11). Therefore, plates should be held for at least 2 weeks prior to being discarded and should be incubated in a humidified environment or sealed to prevent dehydration. Heavy growth of other bacterial species may therefore obscure the presence of the nocardiae on routine culture plates that may be discarded before the nocardial colonies are detected. The presence of nocardiae is frequently noted on fungal cultures that are generally maintained for 4 to 6 weeks (164). Digestion and decontamination procedures used for acid-fast bacilli may be too harsh and render the nocardiae nonviable if the decontamination step is extended; acid-fast bacillus culture therefore should not be the sole method used (149, 150). Selective media may be necessary to enhance recovery of *Nocardia* species and minimize the growth of contaminating organisms. These include a variety of media such as paraffin agar, agar containing antimicrobials, Thayer-Martin medium, and charcoal-buffered yeast extract media (28, 104, 143, 150).

Identification procedures. (i) Biochemical methods. Traditionally the nocardiae were identified to species by using a battery of biochemical tests, including hydrolysis of adenine, casein, tyrosine, xanthine, and hypoxanthine. The two major types of carbohydrate utilization tests used to distinguish among the species of *Nocardia* include those of Gordon et al. and those of Goodfellow et al. (74, 78, 121, 144). However, because of the generally nonreactive nature of most species in these tests and because of the increasing number of described species, species assignment using biochemical methods alone does not provide reliable identification of all the currently recognized species. (See Table 4 for useful tests to establish a tentative identification of the nocardiae, including the major clinically relevant species.)

Biochemically, *N. farcinica* may be differentiated from the other *Nocardia* species by its ability to reach mature growth at 45°C within 72 h, rapid (within 48 h) utilization of acetamide as a sole carbon and nitrogen source, and rapid production of acid in L-rhamnose (214). The majority of strains of *N. farcinica* also produce opacification on Middlebrook agar (62). Although some isolates of *N. asteroides* drug pattern type VI may grow at 45°C, produce acid from L-rhamnose, or utilize acetamide as a sole

carbon or nitrogen source (but not both), mature growth at 45°C is usually not evident as rapidly as that of *N. farcinica* (214). The colony morphologies of these two species are also quite different.

The majority of the isolates of the *N. nova* complex exhibit arylsulfatase activity at 2 weeks, unlike the *N. asteroides* drug pattern type VI or *N. farcinica*, which do not show arylsulfatase activity. The reactions in the majority of other biochemicals, including L-rhamnose and acetamide, are negative (207).

Recently, a novel identification method using an abbreviated battery of tests in the API20C AUX (bioMérieux, Hazelwood, MO) yeast identification system as well as several additional biochemical tests and antimicrobial disk susceptibility was evaluated (111). The authors noted that neither method could identify all of 75 isolates of *Nocardia* representing 10 species to the species level. An algorithm utilizing antimicrobial susceptibility patterns and selected biochemicals (citrate, acetamide, inositol, and adonitol) was able to identify all of the isolates accurately, although some of the species tested consisted of only one isolate (111).

Other investigators have examined commercially available systems for their utility in identifying the nocardiae. Biehle and colleagues evaluated the MicroScan Rapid Anaerobe and *Haemophilus-Neisseria* Identification panels (Dade MicroScan, West Sacramento, CA), which use chromogenic test substrates to detect preformed enzymes, thus eliminating extended incubation times and enabling more rapid results. Agreement between the conventional and enzymatic identification systems was excellent, with isolates identified as *N. asteroides* (we were unable to ascertain which drug pattern groups were included in the definition of *N. asteroides* in those studies), *N. farcinica*, *N. nova*, *N. brasiliensis*, and *N. otitidiscaviarum* (13). Muir and Pritchard also evaluated the bioMérieux ID32 C yeast identification system (bioMérieux, Hazelwood, MO) and found that assimilation results were variable among isolates of the same species and that certain substrates were more discriminatory in determining the species identification (148). In that study, 92 isolates of *Nocardia* spp. were evaluated. Significant variation in assimilation patterns among isolates of *N. farcinica* and *N. transvalensis* and isolates identified as *N. asteroides* was seen, with over half of the isolates tested showing unique patterns (148). Boiron and Provost evaluated the use of the API ZYM system (bioMérieux, Hazelwood, MO) and were able to discriminate isolates of *N. brasiliensis*, *N. otitidiscaviarum*, and *N. transvalensis* and isolates identified as *N. asteroides* from each other (18). None of these commercial systems have been studied extensively, and biochemical identification of *Nocardia* species is nearly impossible given the currently available test methods and the increasing number of new species.

(ii) Chemotaxonomic methods. Whole-cell hydrolysates examined for the presence of meso-diaminopimelic acid and specific carbohydrates are useful tools in the identification of the nocardiae in laboratories that can perform these tests (78). Evaluation of the chromatographic patterns of *p*-bromophenacyl esters of mycolic acids by HPLC may be helpful in identifying isolates resembling *Nocardia* to the genus level. Butler et al. (29) found that the evaluation of the mycolic acid peaks and retention times enabled separation of the nocardiae from mycobacteria and other related aerobic actinomycetes, such as *Rhodococcus* spp. However, most species of the nocardiae show similar mycolic acid patterns by HPLC and are

difficult, if not impossible, to characterize to species.

Gas-liquid chromatography of the short-chain fatty acids has also been used for identification of *Nocardia*. The fatty acid esters released on pyrolysis gas chromatography of mycolic esters consist of 12 to 18 carbons which may be saturated or unsaturated (24, 70, 120, 121, 145). It is not possible to separate the nocardiae from *Mycobacterium* and *Corynebacterium* based on cell wall characteristics alone (145).

McNabb et al. studied the fatty acid compositions of 39 type strains and 529 clinical or reference strains, including nine species of *Nocardia* (*N. asteroides*, *N. brasiliensis*, *N. pseudo-brasiliensis*, *N. carnea*, *N. farcinica*, *N. nova*, *N. otitidiscaviarum*, *N. transvalensis*, and *N. brevicatena*). The fatty acid heterogeneity seen among the *Nocardia* spp. improved identification of some species but also necessitated the creation of fatty acid groups of unknown significance. These groups may represent isolates belonging to heterogeneous groups or species that had not been described at the time the study was performed (135).

(iii) Serological methods. A number of serologic tests have been evaluated to determine their applicability for the early diagnosis of nocardial infections. Most methods utilize culture filtrates or processed cell homogenates of isolates of *N. brasiliensis* or isolates identified as *N. asteroides* as sources of the antigens. Early assays utilized immunodiffusion techniques to detect circulating antibodies (159, 163). These assays proved to be nonspecific, showing positive results for patients with mycobacterial disease. They were also proven to be inadequate for diagnosis of disease in patients with a diminished immune response (16, 85).

Complement fixation tests were also shown to be nonspecific but were able to detect antibodies in most of the serum samples tested from immunocompromised patients (177). Stevens et al. showed that the level of antibody response seemed to be related to the stage of the infection, with higher titers seen early in the disease or, in one case, before the appearance of symptoms (184).

Immunoblot and enzyme-linked immunosorbent assays which detect antibodies to specific high-molecular-weight proteins that appear to be common in various *Nocardia* and *Actinomadura* species have been developed. These antigens do not react with antibodies produced in response to *Mycobacterium tuberculosis* infections (4, 5, 170, 185). Using this information and a Western blot (immunoblot) assay with monoclonal antibodies, Boiron et al. were able to detect immunologic responses to the nocardiae in immunosuppressed patients (18, 21).

Kjelstrom and Beaman evaluated the use of a battery of antigens and a variety of serologic methodologies for detection of antibodies produced in a murine model against isolates identified as *N. asteroides*. Those authors concluded that multiple antigens must be used to optimize the sensitivity and specificity of serologic tests for diagnosis of nocardial disease (112).

None of these serologic methods have been evaluated extensively in recent years. It appears that the utility of a single serologic methodology for the detection of nocardial disease may be limited by the variety of species now known to cause infections and the potential lack of sensitivity for detection of an antibody response in immunocompromised patients.

Antimicrobial susceptibility testing for genus/species identification. Species level identification does predict susceptibility for several species, including *N. brasiliensis*, *N. pseudo-*

brasiliensis, and *N. farcinica*. However, currently many taxa, such as the *N. nova* complex, the *N. transvalensis* complex, and the *N. oitidiscaviarum* complex, are considered to include several species or undesigned taxa.

Generally, only small numbers of some of the species within the complexes have been studied, and thus as new strains are identified, the currently accepted phenotypic descriptions, including antimicrobial susceptibility patterns, may not be valid. This is well illustrated within the *N. nova* complex. Currently this complex is composed of *N. nova*, *N. africana*, *N. veterana*, *N. kruczakiae*, and possible other, yet-unnamed species. With the exception of *N. nova*, only a few isolates of each of these species have been studied in detail, including antimicrobial susceptibility testing. Therefore, until susceptibility testing of large numbers of each of the species to allow for generalization of susceptibility patterns is reported, antimicrobial susceptibility testing of all isolates of *Nocardia* is warranted.

Molecular identification of the nocardiae. It is clear from the previous discussion that molecular methods have had an enormous impact on the taxonomy of *Nocardia*. Analysis of gene sequences has increased understanding of the phylogenetic relationships of *Nocardia* and related organisms and has resulted in the recognition of numerous new species. Molecular techniques have made identification of many species of *Nocardia* more rapid and precise than is possible with phenotypic techniques.

(i) DNA probes. Although DNA probe technology has not yet been made commercially available, more than a decade ago Brownell and Belcher constructed two probes that hybridized with 31% of the *N. asteroides* strains in a reference collection without cross-hybridization with related *Actinomycetales*. However, the investigators abandoned their screening because it was anticipated that more than one strain of *N. asteroides* would be necessary to develop a specific diagnostic probe, again clearly highlighting the heterogeneity of the genus *Nocardia* (26).

Later, Laurent and colleagues used ribotyping as a taxonomic tool in the evaluation of strains of the *Nocardia asteroides* complex and related strains. The probe used was obtained by PCR from DNA extracted from the type strain *N. asteroides* ATCC 19247, a sequence that partially codes for 16S rRNA. Four ribotypes were confirmed in their study: two related to *N. asteroides* sensu stricto, one related to *N. farcinica*, and one related to *N. nova* (118). Ribotyping using a digoxigenin-labeled cDNA probe was subsequently used both taxonomically and as an epidemiologic subtyping tool in distinguishing between *N. farcinica* and the other members of the *N. asteroides* complex (141). Other investigators have described the use of an in situ hybridization technique to detect nocardial 16S rRNA by using a *Nocardia*-specific digoxigenin-labeled probe in specimens from the cerebral cortices of monkeys experimentally infected with *N. asteroides*. Results from these studies suggested a possible association between the presence of *Nocardia* and neurodegenerative disorders (32).

(ii) PCR and PCR-RFLP molecular analysis (PRA). The PCR, alone and in combination with other molecular methods, was one of the first molecular techniques to be used for the characterization of isolates belonging to the genus *Nocardia* or for the identification of *Nocardia* species. In 1999, Laurent et al. described a PCR method using *Nocardia*-specific primers to

distinguish isolates of *Nocardia* from those belonging to other genera of actinomycetes (119). In 2004, Brown et al. used *N. farcinica*-specific PCR primers for identification of members of this species (25).

PRA techniques involve PCR amplification of portions of the *hsp65* gene or the 16S rRNA gene and subsequent digestions with specific restriction endonucleases for each gene. The restriction digest is then electrophoresed on an agarose gel, resulting in a restriction fragment length polymorphism pattern, which may be species specific. PRA techniques take advantage of the variations in gene sequences of species within a genus and of the presence or absence of restriction endonuclease recognition sites within variable regions of the gene.

In 1994, Lungu et al. described a method using PRA of the *groEl* gene to distinguish *Nocardia* isolates from those belonging to the genus *Mycobacterium* (131). In 1995, Steingrube et al. described a PRA method using amplification of a 441-bp region of the *hsp65* gene that allowed identification of 12 species and taxa of *Nocardia* (including isolates belonging to the drug pattern types described by Wallace et al.) (181, 213). Refinement of this procedure using an alternative restriction endonuclease eliminated the intraspecies heterogeneity seen with the original method (183). In 2000, a PRA procedure using a 999-bp region of the 16S rRNA gene was described by Conville et al. (43). This technique allowed differentiation of most of the species of *Nocardia* commonly isolated from clinical specimens. Comparison of identifications obtained using PRA of both the *hsp65* and 16S rRNA genes showed that some isolates gave identical groupings with both genes. However, in some cases, PRA results obtained with the two genes did not correlate with each other; subsequent 16S rRNA gene sequencing of these isolates showed them to be members of undescribed species (43).

As more clinical isolates are tested by PRA methods, it appears that some species show varying degrees of sequence heterogeneity as shown by the variety of RFLPs produced by members of that species. These variant patterns occur when base changes exist within a restriction endonuclease recognition site. One must be careful not to assume that a variant RFLP pattern represents a new species; single base changes within a recognition site, or a single base change that creates a recognition site, produce a change in the RFLP but may not represent a significant difference in gene sequence.

If the *Nocardia* species being tested by PRA methods contains multiple copies of the gene being studied, and if those multiple copies have sequences that vary within a recognition site, the resulting RFLP will show more bands than expected for a species (45).

Another PCR-based assay has been evaluated with the use of arbitrary primers. The use of a PCR-RAPD fingerprinting technique has been evaluated for its ability to distinguish among various species of *Nocardia*. This technique involves PCR of extracted DNA with a set of arbitrary primers and gel electrophoresis of the resulting fragments. The results showed specific banding patterns for 13 type strains of *Nocardia* (not all of which were clinically significant) and multiple banding patterns for isolates identified as *N. asteroides*, *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis*, suggesting that these species may be heterogeneous (89).

(iii) **DNA sequencing.** The availability of gene sequencing has revolutionized the taxonomy of the genus *Nocardia* and has become an invaluable tool for the identification of clinical isolates of this genus. For laboratories with molecular capabilities, gene sequencing provides rapid and usually reliable identifications for most *Nocardia* isolates. It has become clear that the number of distinct taxa and the number of species capable of causing human disease were underestimated when identification relied solely on phenotypic characteristics (157, 167).

The 16S rRNA gene is most often used for species identification; in fact, GenBank, the large public database maintained by the National Center for Biotechnology Information of The National Library of Medicine, contains over 90,000 16S rRNA gene sequences for numerous genera of bacteria (37). For *Nocardia* species the 16S rRNA gene is highly conserved, containing extensive regions that are identical for all species as well as variable regions, which are species specific. One such variable region exists within the first 500 bp of the gene (43) and has made partial 16S rRNA gene sequencing (500 bp) a fairly reliable method for molecular identification of some members of this genus.

Patel et al. examined the correlation of identifications obtained using 500-bp sequences of the 16S rRNA genes of 61 isolates representing 11 species of *Nocardia* with identifications obtained using the combination of *hsp65* PRA, biochemical tests, antibiotic susceptibility tests, and cell wall fatty acid studies (157). Isolates determined to belong to a particular species by PRA, phenotypic tests, and cell wall studies were, in most cases, most closely related to the type strain of that species when 500-bp sequences were evaluated using the GenBank database. Neither complete 16S rRNA gene sequencing nor DNA-DNA hybridization was performed to test the validity of these identifications. Sequence heterogeneity within the partial 16S rRNA region examined was noted among clinical isolates assigned to *N. nova*, *N. otitidiscaviarum*, and the *N. transvalensis* complex. The assessment of the significance of these heterogeneities would require further studies by DNA-DNA hybridization of the isolates to the type strains for the species to which they are presumed to belong.

Cloud et al. reported a comparison of identifications obtained using partial 16S rRNA gene sequences and sequences of a 999-bp region of the gene for 94 patient isolates representing 10 species. This study showed 72% correlation between identifications obtained using partial 16S rRNA gene sequences and phenotypic methods and 90% correlation with sequence results for the 999-bp region. In this study some clinical isolates of *N. abscessus* or *N. asteroides* drug pattern type IV as determined by partial 16S rRNA gene sequencing showed more dissimilarity to the type strains of those species when a longer region of the gene was amplified. In these instances the longer sequences gave a more precise identification than did the shorter sequences (39).

In 2003, Roth et al. sequenced the complete 16S rRNA gene sequences (1,494 bases) of 63 *Nocardia* isolates representing 25 species. Those authors noted both high levels of sequence similarity among some isolates known to be distinct species and significant sequence heterogeneity among isolates of the same species. Their studies also found a number of isolates with unique 16S rRNA gene sequences; these may prove to belong to as-yet-

undescribed species (167).

Since 16S rRNA gene sequencing is frequently used for species identification, isolates with unique 16S rRNA gene sequences have been designated distinct species, resulting in a considerable increase in the number of recognized species of *Nocardia*. Many of these species have been determined to be clinically significant. Some new species have been described based on recovery of a small number of isolates or even a single isolate. In these cases the phenotypic data supplied may or may not represent the typical reactions for this species. Studies on additional isolates of these species will be required to establish the frequency of occurrence of various phenotypic characteristics within members of the species, as well as the geographic distribution and clinical significance of these species.

Recently, Wauters and colleagues studied the species distribution of the nocardiae in a large number of clinical samples in Belgium by using 16S rRNA gene sequencing. The investigators recovered 86 strains of *Nocardia*; among these, 83 (96%) of the strains belonged to only six *Nocardia* species: *N. farcinica* (44%), *N. nova* (22%), *N. cyriacigeorgica* (15%), *N. brasiliensis* (6.9%), *N. abscessus* (5.8%), and *N. paucivorans* (2.3%) (218).

There is little information in the literature regarding sequence analysis of other genes that may be useful for identification of *Nocardia* species. Although PRA of the *hsp65* gene has been used for identification purposes, at this time no sequence information for this gene for *Nocardia* species is available in public databases. The *secA1* gene, which codes for an essential secretory protein, SecA, has shown potential for identification of these organisms, but, like for *hsp65*, sequences are not available in public databases (P. S. Conville, A. M. Zelazny, and F. G. Witebsky, Abstr. 105th Gen. Meet. Am. Soc. Microbiol., abstr.C-152, 2005). The *secA1* gene showed more interspecies heterogeneity than did the 16S rRNA gene, and intraspecies microheterogeneities could be resolved when the DNA sequence was translated into the corresponding amino acid sequences. Clarridge noted that genes encoding proteins may show less base conservation than the 16S rRNA gene (37), so the broad applicability of *secA1* and *hsp65* sequencing for species identification remains to be determined.

One of the most important components in the identification of *Nocardia* isolates by gene sequence analysis is the quality of the databases used for sequence comparisons. Various sequence databases are available for identification purposes. These include MicroSeq 500 (Applied Biosystems), along with the general GenBank database and the National Center for Biotechnology (NCBI). There has been significant criticism of the GenBank database because sequences submitted to the database are not checked for accuracy or for appropriate species assignment (142, 156, 157, 197). Because of the rapidly changing *Nocardia* taxonomy, the species associated with some sequences may not have the correct nomenclature based on today's standards. In addition, sequences are present for "species" that have not been officially described, and some sequences which are identified merely as "*Nocardia* species" are included. Due to the relative inaccuracies that existed in sequencing technology before the mid-1990s, some sequences in the database have significant numbers of ambiguous bases. A proprietary database, the MicroSeq 16S rDNA Bacterial Identification System (Applied Biosystems, Foster City, CA) has enabled species identification for many clinical strains of *No-*

cardia. However, this database remains inadequate, as only one entry per species (the type strain sequence from the ATCC) is available for comparison of most species. Users of this database, however, have augmented it with sequences of their own isolates (39). The Ribosomal Differentiation of Microorganisms (RIDOM) database is a controlled database that verifies the sequences and species assignments of submitted sequences. This database includes 500-bp sequences for *Nocardia* species; however, there are no plans to make this database publicly accessible at this time.

As with any method of assigning a species identification to an isolate, sequence matches obtained through databases must be carefully analyzed. The relative similarity of the most likely matches should be considered; seemingly inappropriate entries (for example, the presence of an *N. otitidiscaviarum* sequence among numerous *N. farcinica* sequences) may represent a misidentification of the submitted sequence. Ideally, the sequence of an unknown isolate should be closely matched to the sequence of a type strain, and the most closely matching sequence will contain no ambiguous bases.

There is no consensus on how similar the 16S rRNA gene sequences of *Nocardia* isolates must be in order to consider them as belonging to the same species. It is well known that nocardiae are relatively homogeneous in regard to their 16S rRNA gene sequences; isolates which have been shown to be distinct species by DNA-DNA hybridization can have as much as 99.8% sequence similarity (41, 44). Fox et al. commented that bacterial isolates that have essentially identical 16S rRNA gene sequences in most cases belong to the same species. However, there are some species of bacteria that show 100% sequence similarity and have been shown to be distinct species by phenotypic characteristics and DNA-DNA hybridization. Therefore, even a small percentage of sequence diversity between isolates may be indicative of distinct species; in these cases only DNA-DNA hybridization (the current "gold standard") can prove that the isolates belong to different species (63).

A recent study reported that some species of *N. nova* contain multiple 16S rRNA gene sequences, which may or may not have similar sequences within their variable regions. It remains to be determined if isolates with multiple different 16S rRNA genes can be misidentified using sequencing of this region (45).

(iv) Pyrosequencing. Recently, the technique of pyrosequencing has been evaluated for use in the identification of various clinically relevant *Nocardia* species. Pyrosequencing involves preliminary amplification of a specific gene region and subsequent short-read sequencing of a section of that region which includes variable bases. Touhy et al. utilized pyrosequencing of the hypervariable region of the 16S rRNA gene and identified isolates by comparison of resulting sequences contained in GenBank and in an in-house database. Thirty-eight of 45 identifications obtained by pyrosequencing were identical to identifications obtained for those isolates by PRA of the *hsp65* gene (M. J. Touhy, J. J. Farrell, B. A. Brown-Elliott, L. Mann, R. W. Wilson, R. J. Wallace, Jr., G. S. Hall, and G. W. Procop, Abstr. 104th Gen. Meet. Am. Soc. Microbiol., abstr. C-017, 2004).

(v) Ribotyping. Ribotyping has been used to differentiate a limited number of *Nocardia* species. This technique involves extraction of the genomic DNA and subsequent digestion with a restriction endonuclease, electrophoresis of the digest, and

the preparation of a Southern blot using a labeled DNA probe, resulting in a banding pattern specific for individual species. One study using this technique showed various banding patterns for a number of isolates identified as belonging to the *N. asteroides* complex (118). Additionally, McNeil et al. showed a clinical isolate identified as *N. farcinica* by phenotypic methods to have a ribotype identical to that obtained with the type strain of *N. farcinica* but different from those obtained with the type strains of *N. nova* and *N. asteroides* (141).

The use of a PCR-RAPD fingerprinting technique has been evaluated for its ability to distinguish among various species of *Nocardia*. This technique involves PCR of extracted DNA with a set of arbitrary primers and gel electrophoresis of the resulting fragments. The results showed specific banding patterns for 13 type strains of *Nocardia* (not all of which were clinically significant) and multiple banding patterns for isolates identified as *N. asteroides*, *N. nova*, *N. pseudobrasiliensis*, and *N. transvalensis* (89), suggesting that these species may be heterogeneous.

(vi) Direct detection in paraffin-embedded tissue and clinical samples. Wada and colleagues accurately diagnosed nocardiosis in a pathological specimen of culture-negative granulomatous pleura tissue by a PCR-based 16S rRNA gene sequencing analysis (202). After DNA extraction using a procedure described by Laurent et al. (119) and a modification for the paraffin wax removal from embedded sections, an analysis was performed that combined genus-specific primers reported by Laurent et al. (119) and an additional set of primers from *N. asteroides* (accession no. X80606) designed by the authors. The resulting combined sequence of the two products was 971 bp in length. This sequence matched the 16S rRNA gene sequence of *Nocardia* sp. strain IFM 0860, a microorganism isolated from soil. *Nocardia* sp. strain IFM 0860 was later included in a taxonomic study and classified as *N. asiatica* (92).

Couple et al. also described a 16S PCR-based assay for the direct detection of *Nocardia* spp. in clinical specimens by using the primers developed by Laurent et al. (47, 119). Importantly, the extraction procedure used was chosen because of the extensive validation of the MTB respiratory specimen preparation kit (Roche, Meylan, France) for *M. tuberculosis* DNA. The applicability of this assay was confirmed by using 18 samples from patients with nocardiosis diagnosed by conventional cultures and 20 clinical samples from patients with confirmed tuberculosis used as negative controls. This procedure involves PCR amplification of a 590-bp region of the 16S rRNA gene with *Nocardia*-specific primers and Southern blotting using a probe specific for this gene region. Using this method, the authors were able to detect five species of *Nocardia* directly from 18 clinical samples. Seeded samples were tested to evaluate the sensitivity of this method; 10^3 organisms were detectable by gel electrophoresis after amplification alone. Use of the probe increased the sensitivity to allow detection of 1 CFU per reaction (47).

Antimicrobial Susceptibility Testing for Predicting Clinical Response

In 2003, the CLSI published the first approved recommendations for antimicrobial susceptibility testing of aerobic actinomycetes, including the nocardiae (152). The preferred method is

broth microdilution using standard twofold dilution of antimicrobials in cation-adjusted Mueller-Hinton broth. This method has been detailed elsewhere (23), and MIC panels are now available commercially. The recommended primary 10 antimicrobials for susceptibility testing are amikacin, amoxicillin-clavulanic acid, ceftriaxone, ciprofloxacin, clarithromycin, imipenem, linezolid, minocycline, sulfamethoxazole or TMP-SMX, and tobramycin. Secondary agents recommended are cefepime, cefotaxime, doxycycline, gentamicin, gatifloxacin, and moxifloxacin (152).

Performance and interpretation of antimicrobial susceptibility testing with the nocardiae require skill acquired through experience with the test method and knowledge of the differing susceptibility patterns of various species. Laboratories that infrequently encounter *Nocardia* are urged to refer these organisms to qualified laboratories. For laboratories that prefer to perform in-house susceptibility testing, initial validation and regular evaluation of test performance should be done to demonstrate continued proficiency. Antimicrobial susceptibility testing by broth microdilution is not without its own pitfalls. Isolates such as those in the *N. nova* complex may grow poorly and make reading at the standard 72-h interval difficult. Incubation for up to 5 days may be necessary; if no growth is present at 5 days, the panel should be reinoculated using a slightly heavier inoculum. Also, unless the growth in the control well is adequate, interpretation of some MICs, especially those of the sulfonamides, may be indeterminate.

Prior to the development of the broth microdilution technique for antimicrobial susceptibility testing of the *Nocardia*, agar disk diffusion using commercially available antibiotic disks was used in many clinical laboratories (210, 212). Aminoglycosides and ciprofloxacin usually gave sharp zones of inhibition, whereas the tetracyclines, beta-lactams, and sulfonamides had hazy or partial zones of inhibition, making reading of the end point difficult (215). In addition, for many of the newer agents (e.g., clarithromycin and linezolid) no correlation studies of this method with agar or broth microdilution are available.

Using susceptibility testing results, Wallace et al. noted that isolates identified as *N. asteroides* showed multiple susceptibility patterns. In 1988, six major groups (types I to VI) were defined. A summary of these drug patterns is given in Table 3 (213).

One of the most recent antimicrobial agents evaluated with strains of *Nocardia* spp. is the oxazolidinone linezolid. Linezolid is the first antimicrobial (other than the sulfonamides) to be active in vitro against all clinically significant species of the genus *Nocardia*. In a study by Brown-Elliott et al., of 140 isolates, MICs for all species studied were ≤ 8 $\mu\text{g/ml}$. The MIC₉₀ for all species, including *N. asteroides* drug pattern type VI, *N. farcinica*, *N. nova*, *N. brasiliensis*, *N. pseudobrasiliensis*, the *N. transvalensis* complex, *N. otitidiscaviarum*, *N. paucivorans*, *N. abscessus*, and *N. veterana*, was ≤ 4 $\mu\text{g/ml}$ (27).

Although broth microdilution is now the CLSI recommended method of antimicrobial susceptibility testing of the nocardiae, other methods have been evaluated previously, including agar dilution and the Etest (AB Biodisk, Solna, Sweden), an agar diffusion method that yields a quantitative MIC result. Biehle et al. noted that with close attention to inoculum standardization and cautious interpretation criteria after more than 72 h of incubation, the Etest was a suitable alternative method for susceptibility testing of the nocardiae (14). However, few other correla-

tion studies with the broth microdilution method have been performed. Like with broth microdilution, growth and interpretation of results for some species with these methods may be difficult and require skill and experience.

Thus, antimicrobial susceptibility patterns can provide tentative species identification within the genus *Nocardia*; however, like for biochemical and chemotaxonomic methods, these results can be inexact, and identification of *Nocardia* to species or complex requires molecular confirmation.

Clinical Outcomes Related to In Vitro Susceptibility Testing

The relationship between in vitro susceptibilities and in vivo results based on clinical studies is not well established for nocardiosis. There are no published prospective clinical trials, in part because of the rarity of the disease. However, in the preantibiotic era, pulmonary nocardiosis was considered a 100% fatal condition. The first cure of clinical disease was with a sulfonamide, with this class of drugs remaining the drug of choice for almost 50 years. With the availability of modern drugs and susceptibilities, there are few reports of treatment failures. Our laboratories have performed susceptibility testing on *Nocardia* for almost 30 years, with innumerable clinical consultations. With the exception of nocardial brain abscesses (46), our perception is that modern therapy based on in vitro susceptibility results is highly effective, although we recognize the paucity of published data to support this (8, 123).

Does every situation require identification to species level?

Determination of the species of *Nocardia* is useful to help define the spectra of disease caused by species and the relative pathogenicities of the various species for different patient groups. Currently only a few species, such as *N. brasiliensis*, *N. farcinica*, and *N. pseudobrasiliensis*, can be reliably identified by biochemical methods, including hydrolysis, colonial morphology, and antimicrobial susceptibility patterns. Identification of *Nocardia* to species level is becoming increasingly difficult without the use of molecular techniques. Conventional methods of identification appear to be unreliable for species level identification of many species of *Nocardia* due to the small number of discriminatory tests available and the expertise needed to interpret these tests (43). Compared to these methods, molecular testing by *hsp65* PCR and 16S restriction enzyme analysis (PRA) appears to be an improvement and recognizes >90% of currently recognized clinical species. Not all restriction profiles are unique, and some discrepancies may occur with results from sequence analysis, especially with the new described species that may not be in PRA databases.

For laboratories that do not have access to sequencing methods, identification to complex level for some clinically significant species, such as the *N. nova* complex, *N. otitidiscaviarum* complex, *N. transvalensis* complex, and *N. brevicatena/paucivorans* complex of *Nocardia*, may be an acceptable compromise (45, 162). For other *Nocardia* spp., including the newly described species, molecular identification (i.e., sequence analysis) is essential for species level identification.

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