Nocardia cyriacigeorgica is an emerging pathogen in the United States

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Abstract

*Nocardia cyriacigeorgica* is recognized as an emerging pathogen in many parts of the world. We present the first case description of invasive *N. cyriacigeorgica* pulmonary infection in the USA identified to the species level by 16S rRNA and *hsp65* sequence analysis. A subsequent retrospective molecular screening of recent *Nocardia* clinical isolates at our New York City medical center yielded an additional six *N. cyriacigeorgica*. Because routine laboratory algorithms for the phenotypic identification of *Nocardia* species are limited in practice, the true prevalence of *N. cyriacigeorgica* infections may be greater than currently appreciated. Indeed, we present evidence confirming that *N. cyriacigeorgica* is coincident with the unofficial species designation *Nocardia asteroides* complex antimicrobial susceptibility pattern Type VI and distinct from the *N. asteroides* sensu stricto strain ATCC 19247. As nocardial species identity can predict antimicrobial susceptibility and guide clinical management, we offer simplified phenotypic and molecular protocols to assist the identification of *N. cyriacigeorgica*. 
**Introduction**

Nocardiae are aerobic actinomycetes ubiquitously found in soil and aquatic habitats. *Nocardia* are beaded, branching gram-positive rods that are partially acid-fast and are generally slow growing (6). Approximately 50 *Nocardia* spp. have been described to date, about 30 of which are known to cause human disease (6). Infections due to *Nocardia* spp. are generally acquired through inhalation or percutaneous inoculation from environmental sources. Nosocomial transmission has also been reported (12, 16, 36). Those individuals mainly affected are immunocompromised (6, 21), although cutaneous infections may also be seen in immunocompetent hosts (6). Mortality appears to correlate with the causative species, the site of infection, and can be as high as 50% in patients with disseminated disease (15, 21, 30).

Laboratory identification of nocardiae and their differentiation from nontuberculous mycobacteria (NTM) can be challenging and time-consuming using routine culture and chemotaxonomic methods. Indeed, most *Nocardia* fail to react in many of the standard biochemical reactions utilized in clinical microbiology laboratories, thus oftentimes rendering it extremely difficult to make a precise species level determination. To address this problem, Wallace *et al.* reported on species-indicative differences in antimicrobial susceptibility patterns that could be used to segregate many of the strains previously identified as *Nocardia asteroides* or as a species of the former *N. asteroides* complex (34). Included in these six designations are the *Nocardia nova* complex (type II), the highly antimicrobial resistant *Nocardia transvalensis* complex (type IV) and *Nocardia farcinica* (type V), as well as the unnamed species with drug pattern Type VI (reviewed in ref. 1). Unfortunately, this methodology based on antimicrobial susceptibility patterns is not well-suited for the routine clinical microbiology laboratory as it is technically demanding, time-consuming, labor-intensive, slow to results, and does not always provide a definitive identification because patterns can be shared between species (34). Notably, the *N. asteroides* sensu stricto species (represented by strain ATCC 19247T) has not been isolated clinically nor is it included in any drug pattern group (6). More recently, *Nocardia* species identification and taxonomy have been improved by molecular methods such as 16S rRNA and *hsp65* gene sequence analysis (6). In general, these procedures are rapid, accurate, and reproducible and can better discriminate among strains of actinomycetes than is possible with phenotypic methods (8). However, there have been few reports on the application of these
methodologies in clinical microbiology laboratories for the diagnosis of *Nocardia* infections (5, 10, 23, 31).

Recent reviews of collections of *Nocardia* clinical isolates by genotypic methods have found that a significant proportion, originally identified by routine phenotypic and chemotaxonomic methods, belong to a new species, *Nocardia cyriacigeorgica* (18, 27, 29, 35). This species was first described in 2001 (37) and strains of *N. cyriacigeorgica* have since been recovered as the etiologic agent of human infection in Western Europe, Greece, Turkey, Japan, Thailand, and Canada (2, 4, 7, 8, 11, 17, 18, 22, 27, 35, 38). Most cases of infection have occurred in the context of HIV-related or iatrogenic immune suppression. However, only a few complete clinical histories of *N. cyriacigeorgica* infection have been published to date (2, 4, 11, 22).

At present, *N. cyriacigeorgica* per se has not been described as a cause of infection in the USA. However, a growing consensus of opinion holds that the species *N. cyriacigeorgica* is coincident with strains previously classified as having a Type VI drug pattern (6, 11). In addition to sulfonamide susceptibility, Type VI strains are generally susceptible to broad-spectrum cephalosporins, amikacin, imipenem, and linezolid but resistant to penicillins, clarithromycin, and ciprofloxacin. The Type VI antibiogram is similar to that of most reported *N. cyriacigeorgica* strains with the exception of a few strains of *N. cyriacigeorgica* that have been reported to be susceptible to ciprofloxacin (4, 38). *N. cyriacigeorgica* and Type VI strains also share identical partial 16S rRNA gene sequences (6). Most importantly, Type VI strains appear to account for up to 35% of *Nocardia* strains recovered from patients in the southern USA (34). The species *N. cyriacigeorgica* may therefore be a significant unrecognized cause of disease in the USA.

In this study, we report on the application of molecular methods to identify *N. cyriacigeorgica* as the causative agent of a case of atypical pneumonia in a heart transplant recipient. We also describe the recent prevalence and basic clinical histories of infection by this species at a New York City medical center. We make note of *N. cyriacigeorgica* intra-species genotypic and phenotypic variability as well as provide key characteristics to guide *N. cyriacigeorgica* species level identification and differentiation from other *Nocardia* species. Evidence is provided to support the recognition of Type VI strains in the USA as *N. cyriacigeorgica* and the distinction of *N. cyriacigeorgica* from the representative laboratory strain of *N. asteroides* (ATCC 19247).
The Case

The index patient was a diabetic 55-year-old caucasian male heart transplant recipient who presented with a several day history of fatigue, productive cough, fever (38.3°C), and abdominal pain. At the time of presentation, the patient’s immunosuppressive regimen to prevent transplant rejection consisted of cyclosporine, mycophenolate-mofetil, and prednisone. The initial clinical laboratory work-up demonstrated leukocytosis with neutrophilia, lymphopenia, accelerated erythrocyte sedimentation rate, and an elevated lactate dehydrogenase level (822 U/L). Imaging studies revealed nodular opacities in the right upper and lower lobes and a dense consolidation in the left lower lobe (Figure 1A and B). Antimicrobial prophylaxis at admission consisted of atovaquone, valganciclovir, and nystatin mouthwash. Sputum and blood cultures, as well as serum cryptococcal and aspergillus galactomannan antigen tests, were negative. The differential diagnosis included atypical/fungal pneumonia and post-transplant lymphoproliferative disorder. Empiric antimicrobial therapy with ceftriaxone, azithromycin, and voriconazole was initiated. Over the following week the patient displayed increasingly productive cough, intermittent fever (up to 40°C), and persistence of radiological findings.

From day 9 of admission, branching gram-positive, beaded, partially acid-fast filaments were identified by direct microscopic analysis of sputum, bronchoalveolar lavage, and lung biopsy samples. Histopathological examination showed necrotizing inflammation and similar gram-positive, beaded microorganisms with branching filaments (Figure 1C). Heavy growth of a chalky white microorganism was apparent following 1 day of incubation on several solid growth media (n = 8 separate patient specimens were culture positive). Small white colonies were clearly visible after 2 days incubation following subculture. Routine phenotypic test results were characteristic of N. asteroides complex (partially acid-fast, resistant to lysozyme, and negative for the decomposition of casein, tyrosine, and xanthine, as well as negative for starch utilization) and concurrent 16S rRNA and hsp65 gene sequencing identified the bacterium as N. cyriacigeorgica.

From these results the patient’s antimicrobial regimen was adjusted to trimethoprim-sulfamethoxazole (TMP-SMX), and imipenem-cilastatin. Specific anti-nocardial therapy resulted in the rapid alleviation of symptoms and improvement of radiological findings. Antimicrobial susceptibility testing on a representative isolate showed a N. asteroides drug Type VI pattern (34); susceptible to amikacin, cefotaxime, ceftriaxone, gentamicin, imipenem, linezolid, meropenem, minocycline, sulfamethoxazole, sulfisoxazole, and tobramycin but resistant to augmentin,
ciprofloxacin, clarithromycin, gatifloxacin, and kanamycin. The patient was later discharged, receiving intravenous ceftriaxone and vancomycin for 2 weeks. Oral TMP-SMX was administered for an additional 6 months. The patient recovered and remained relapse-free after 15 months. This case presentation is consistent with previous reports noting the heightened risk of serious nocardial infections in transplant patients receiving immunosuppressive therapy (14, 21, 26).

Methods

Nocardial Phenotype-based Identification

Initial specimen handling and the identification of microorganisms as members of the genus *Nocardi*a followed standard laboratory protocols (1, 20). Additional biochemical testing (metabolism of acetamide, glucose, esculin, maltose, rhamnose, trehalose, and urea [Becton, Dickinson and Company (BD); Sparks, MD]; arylsulfatase activity [Remel; Lenexa, KS]; pyrrolidonyl arylamidase (PYR) and α-glucosidase activity [API Coryne, bioMérieux, France]) was performed as per the manufacturers’ recommendations, with the exception that tests requiring an overnight incubation were read after 48-72 h to compensate for the slower growth rate of *Nocardia*. Suspensions for inoculation of the API Coryne strips were vortexed with silicate beads to achieve homogeneous mixtures. Testing for growth at 45°C after 3 days was performed on Sabouraud (SAB) dextrose agar (1). Over the time period from 2000 to 2006, there were 37 patients diagnosed with nocardiosis at our institution. From these, there were 27 patient isolates available for retrospective analysis.

Antimicrobial Susceptibility and Synergy Testing

For a subset of the isolates, initial antimicrobial susceptibility testing was done by disk diffusion and/or broth microdilution methodologies at the Mycobacterial/Nocardia Research Laboratory, University of Texas Health Center. Additional antimicrobial susceptibility and synergy testing on 15 cm Mueller-Hinton agar plates was performed as described previously (3). Briefly, amikacin (30 µg), imipenem (10 µg), and meropenem (10 µg) containing disks (BD) were placed at an adequate distance apart and incubated for 10 days. For synergy testing, discs were spaced 3.5 cm apart. Zones of inhibition were recorded at 24 h intervals for 4 days. Etests (AB BIODISK; Sweden) for the carbapenems were read at 24 h intervals for 4 days.
16S rRNA and hsp65 PCR and Sequence Analysis

Samples from pure subcultures of various nocardial strains were thermolysed (80°C for 30 min) and acted as the source of DNA template for PCR. The near complete length of the 16S rRNA gene (1512 bp) of each test isolate was targeted for PCR amplification using the universal bacterial primers; 16SF: 5’-AGA GTT TGA TCM TGG CTC AG–3’ (*Escherichia coli* positions 8-27 [GenBank nucleotide accession number J01859]) and 16SR: 5’- TAA GGA GGT GAT CCA RCC GCA–3’ (*E. coli* positions 1541-1522). Each PCR reaction was prepared with 25 µl of PCR Master Mix (Roche Diagnostics, Indianapolis, IN), 18 µl of water, 2.5 µl of dimethyl sulfoxide, 1 µl of each primer at 20 µM, and 2.5 µl of thermolysate. Each PCR amplification was performed in a MJ Research PTC-200 Peltier Thermal Cycler (Bio-Rad, Hercules, CA) using the following program: an initial denaturation step of 5 min at 94°C followed by 45 cycles of 1 min at 94°C, 4 min at 60°C, and 1 min at 72°C, and ending with a final elongation step for 10 min at 72°C. PCR products and a 100-bp DNA ladder (Invitrogen, Carlsbad, CA) were visualized by 2% agarose gel electrophoresis and ethidium bromide staining. Images were captured using the Gel Doc™ XR (Bio-Rad) digital image capture system and the Quality One 1-D Analysis Software version 4.6.0 (Bio-Rad). The hsp65 gene (441 bp) was amplified in the same manner using primers hsp65F: 5’-ACC AAC GAY GGT GTB TCC AT-3’ and hsp65R: 5’-CTT GTC GAA SCG CAT RCC CT-3’. These primers were adapted from oligonucleotides previously described for the amplification of hsp65 in mycobacteria (32).

For 16S rRNA sequencing, additional universal bacterial primers that anneal to sites internal to the target 16S rRNA amplicon were utilized; 16SiF2: 5’-GTG CCA GCA GCC GCG GTA ATA C-3’ (*E. coli* positions 514-535), 16SiF3: 5’-GGT TAA GTC CCG YAA CGA GCG-3’ (*E. coli* positions 1087-1107), and 16SiR: 5’-GGA CTA CCA GGG TAT CKT AAT–3’ (*E. coli* positions 805-786). The hsp65 amplification primers were used for sequencing. Direct sequencing of PCR fragments was performed at the Columbia University DNA Sequencing Lab (https://www.dnasequencing.hs.columbia.edu) using a BigDye Terminator kit (PE Applied Biosystems) and an ABI 3700 DNA sequencer. The Lasergene program (DNASTAR Inc., Madison, Wis.) was used to analyze the derived sequence data. By this protocol, complete coverage of the 16S rRNA and hsp65 amplicon sequences were acquired for each bacterial strain with one or more sections of overlap. Consensus 16S rRNA and hsp65 sequences for each strain...
were constructed based upon alignments of the sequence data and a careful examination of each
electropherogram trace representation of the data. This strategy was necessary in order to reconcile
any ambiguous bases or conflicting base assignments and to look for consistent double peaks,
which would indicate the presence of one or more 16S rRNA alleles. To identify each microbe to
the species level, sequencing data were queried against previously submitted sequences in the
GenBank database (http://www.ncbi.nlm.nih.gov) using the BLASTN program. A similarity of
>99.5% to the closest relative 16S rRNA sequences was used as the criterion for identification.

Results
16S rRNA and hsp65 Gene Sequence Analysis of Index Case Isolates
A total of 8 isolates were obtained from the index patient and the respective 16S rRNA and
hsp65 gene sequences from each were identical. An ambiguous nucleotide (C/T) was present at
position 448 of the consensus 16S rRNA gene sequence of each strain (Table 2, nucleotide position
numbering as for E. coli [GenBank accession number J01859]). This indicates that N.
cyriacigeorgica may possess more than one copy of this gene as it has been reported for other
nocardiae (6, 9). Comparison of the 16S rRNA and hsp65 gene sequences from each isolate with
GenBank deposits by BLASTN analysis revealed N. cyriacigeorgica strain DSM 44484T to be the
best match for each sequence (>99.5%). Based upon these data, we believed the true identity of the
infecting microorganism to be N. cyriacigeorgica. Isolate 06-51518 originated from a lung biopsy
and was used as the index patient’s representative strain.

Molecular-based Retrospective Review of Clinical Isolates
To estimate the prevalence of N. cyriacigeorgica infections at our institution, we screened a
collection of 27 Nocardia patient isolates acquired over a 6-year period (2000-2006) by sequence
analysis of 16S rRNA and hsp65. Overall, 6 strains were found to be N. cyriacigeorgica (26% of
patients, including the index). These strains were originally identified either as Nocardia sp., N.
asteroides complex, or N. asteroides complex drug pattern Type VI (Table 1), as per the standard
diagnostic work-up and accepted taxonomy at the time of isolation. Each was a confirmed
pathogen by clinical chart review. The 16S rRNA and hsp65 sequences from these strains were
however clearly distinct from the analogous sequences available in GenBank for the N. asteroides
sensu stricto species (represented by the laboratory strain ATCC 19247T). The N. cyriacigeorgica
strains were isolated from respiratory samples (n = 4), a pharyngeal abscess (n = 1), and an orbital
abscesses (n = 1). Similar to prior reports on infections by this species, the six patients exhibited a spectrum of immunocompromising conditions and/or pulmonary disorders that included AIDS, corticosteroid treatment, chronic granulomatous disease (CGD), chronic obstructive pulmonary disease, and interstitial lung disease (Table 1). All but two patients were known to be on corticosteroid treatment. Notably, whereas multiple infections by this species have been described in kidney transplant patients, we now report the first two cases of *N. cyriacigeorgica* infection in heart transplant patients.

We also reexamined the 16S rRNA and hsp65 gene sequences from the *N. asteroides* drug pattern VI type strain ATCC 14759^T_. As others have indicated (6), it also had a comparably high index of similarity (>99.5%) to both our patient strains in these genes, as well as other sequences from *N. cyriacigeorgica* clinical isolates and the type strain DSM 44484^T_ (Table 2). Hence, these data supported previous suggestions that *Nocardia* isolates with a Type VI drug pattern in the USA are actually *N. cyriacigeorgica* (6).

**Genotypic Segregation of *N. cyriacigeorgica***

In comparing the 16S rRNA and hsp65 sequences of the *N. cyriacigeorgica* patient isolates we noted intraspecies microheterogeneity (Table 2). Previously, Roth et al. distinguished two genotypes of *N. cyriacigeorgica* based upon a single nucleotide polymorphism (SNP) at position 1427 of the 16S rRNA gene (29). According to this classification scheme, *N. cyriacigeorgica* strain DSM 44484^T_, the drug pattern VI type strain, and three out of our seven clinical strains belong to genotype I, while four belonged to genotype II. We identified an additional SNP at position 1480 that segregated genotype I, thus allowing us to designate a new genotype III represented by two clinical isolates, one of which is the *N. cyriacigeorgica* strain from the index patient. The *N. cyriacigeorgica* hsp65 sequences were relatively more polymorphic than the 16S rRNA sequences (28) but cosegregated by 16S rRNA genotype (Table 2). The 16S rRNA and hsp65 sequences from each respective strain were then concatenated and subjected to phylogenetic analysis. The resulting tree is shown in Figure 2 and clearly illustrates the three clades.

Interestingly, two of the cases with *N. cyriacigeorgica* infection were brothers, 5 years apart in age, and both suffered from CGD. However, a common source of infection is unlikely as the
strains (00-12607 and 03-70718) were of different genotypes (II and III, respectively) and isolated three years apart. CGD is a known risk factor for infections with *Nocardia* spp. (6).

### Phenotypic Analysis of *N. cyriacigeorgica* Strains

Because many clinical microbiology laboratories do not have the resources to perform gene sequence analysis, we reevaluated and summarized the phenotypic and biochemical profiles of *N. cyriacigeorgica*. Each of the *N. cyriacigeorgica* clinical strains (*n* = 7), as well as the drug pattern VI type strain and the *N. asteroides* ATCC 19247\(^T\) strain, were evaluated in parallel. The type strains of additional clinically relevant *Nocardia* spp. were included in certain evaluations to act as controls.

All *N. cyriacigeorgica* strains (which we henceforth consider to include the drug pattern VI type strain) grew clearly visible individual colonies after 1-2 days of incubation on Columbia 5% sheep’s blood agar plates (BAP), buffered charcoal yeast extract (BCYE) agar, Middlebrook 7H11 agar, (Figure 3A - C), as well as SAB agar (not shown). After three to four days of incubation, the colony morphologies of all *N. cyriacigeorgica* strains began to vary by medium. By day 7, individual colonies on BAP were white to pale yellow, chalky, flat to mildly ruffled, and approximately 3 to 8 mm in diameter. Colonies on BCYE by day 7 appeared similar but had a highly ruffled texture and the colony edges curled up from the agar. In contrast, day 7 colonies on 7H11 formed discrete cream-colored bodies that grew up from the agar with a central indentation. An isolate from the index patient, respectively grown on BAP, BCYE, and 7H11 is shown in Figure 3D-F. When a heavy inoculum from liquid culture was plated on SAB and examined after 4 days the resulting mega-colonies of *N. cyriacigeorgica* were a chalky white to pale yellow color, flat, and relatively smooth on their surface (Figure 3G). Microscopically, colonies showed delicate aerial hyphae with multiple branch points (Figure 3H). The Gram stain appearances from SAB cultures showed gram-positive, beaded, branching filaments after 2 days of growth that fragmented into single, coccobacillary forms by day 7 (Figure 3I and J). After an extended period of incubation all *N. cyriacigeorgica* strains produced an earthy odor that is typical of *Nocardia*. The microscopic and morphologic features of the *N. asteroides* laboratory strain ATCC 19247\(^T\) are illustrated for counterpoint comparison in Figure 4.
All *N. cyriacigeorgica* also formed a cohesive group by chemotaxonomic testing. Each strain demonstrated the expected phenotype of a *N. asteroides* complex species: positive in the lysozyme resistance test and negative for starch utilization, as well as negative for casein, xanthine, and tyrosine decomposition. However, unlike the *N. asteroides* laboratory strain ATCC 19247\(^\text{T}\), all *N. cyriacigeorgica* were negative for urea hydrolysis and grew at 45\(^\circ\)C. Further biochemical profiling was performed according to previously published phenotypic descriptions of *N. cyriacigeorgica* and Type VI strains (6, 37). The results are provided in Table 3. In summary, growth at 45°C, esculin hydrolysis, and \(\alpha\)-glucosidase activity were each positive for the *N. cyriacigeorgica* strains. The fermentation of glucose, maltose, rhamnose, and trehalose, as well as PYR and 14-day arylsulfatase activity was negative. The utilization of acetamide as the sole source of carbon, a test known to be variable in *N. cyriacigeorgica* (25), was only positive for strains 02-50508 and the drug pattern VI type strain. Interestingly, *N. cyriacigeorgica* strain DSM 44484\(^\text{T}\) is also reportedly acetamide test positive and, along with the latter two strains, is included in the genotype I grouping by 16S rRNA (6, 37). A positive acetamide test may therefore distinguish this subgroup of *N. cyriacigeorgica*.

Overall, the combined results of the phenotypic and chemotaxonomic testing of the index case isolates (\(n = 8\)), the other clinical strains (\(n = 6\)), and the drug pattern VI type strain were in accordance with those previously reported for *N. cyriacigeorgica*. As such, these results confirmed the species assignments based on 16S rRNA and *hsp65* sequences and supported prior suppositions that the drug pattern VI type strain is indeed a *N. cyriacigeorgica* (6). Importantly, the *N. cyriacigeorgica* strains together possessed an overall unique pattern of morphologic, microscopic, phenotypic, and biochemical test results as compared to the various *Nocardia* type strains that were evaluated (Table 3 and data not shown), including the *N. asteroides* sensu stricto laboratory strain ATCC 19247\(^\text{T}\) (Table 3 and Figure 4).

### Antimicrobial Susceptibility and Synergy Testing

Amikacin is the most commonly used aminoglycoside to treat severe nocardial infections in the USA and is often combined with a carbapenem such as imipenem (6). Susceptibility of our *N. cyriacigeorgica* strains (\(n = 8\)) to select antimicrobials was assessed by the disk diffusion method. The zones of absolute inhibition ranged from 19 to 23 mm (mean 22 mm) for amikacin, 26 to 32 mm (mean 29 mm) for imipenem, and 11 to 19 mm (mean 16 mm) for meropenem (Figure 5A).
This is consistent with the reported susceptibility of *N. cyriacigeorgica* and Type VI strains (7). To confirm the observed higher susceptibility to imipenem than to meropenem, strains 06-51518 and the drug pattern VI type strain were also tested by Etest. Minimal inhibitory concentrations for strains 06-51518 and the drug pattern VI type strain were 0.5 and 0.75 µg/ml for imipenem, and 4 and 3 µg/ml for meropenem, respectively (Figure 5B). Imipenem therefore appeared to be the more effective carbapenem (by 5 to 6-fold) against *N. cyriacigeorgica* by in vitro testing.

Synergistic effects of aminoglycoside and carbapenem antimicrobials on growth inhibition of *Nocardia* have been reported (13, 19). Based upon the potent in vitro activity of imipenem, we evaluated for synergy between amikacin and imipenem against *N. cyriacigeorgica* by disk diffusion. Indeed, a modestly expanded area of clearance where the zones of inhibition meet for each disk was observed suggesting the existence of a synergistic effect between these antimicrobials (Figure 5A). These results therefore provide supporting in vitro evidence to explain the underlying basis of combination therapy with amikacin and imipenem as an anti-nocardial regimen for serious *N. cyriacigeorgica* infections.

**Discussion**

The incidence of nocardial infections is believed to be on the rise in the USA as a result of a growing immunocompromised population and improved methods for pathogen isolation and molecular identification (6, 37). The number of recognized *Nocardia* species causing infections is also increasing (6, 37). These factors have combined to place ever-greater demands on clinical microbiology laboratories to accurately identify *Nocardia* clinical isolates to the species level. Indeed, accurate and timely identification of nocardiae is important because the pathogenic potential between species varies and because the species identity provides a critical guide for physicians in the choice of targeted therapy owing to species-specific differences in resistance patterns to key antimicrobial agents (15, 21, 30, 33).

Historically, infections with *Nocardia* were associated with very high mortality rates. Treatment of serious nocardial infections with sulfonamides has greatly decreased mortality, and TMP-SMX is the current basis for antimicrobial treatment of nocardiosis in the USA. However, combination treatment is preferred, especially in severe, disseminated, and CNS infections (6). Amikacin plus a beta-lactam (ceftriaxone or imipenem) are typically added to TMP-SMX to ensure the
susceptibility of all *Nocardia* spp. to at least two antimicrobials. Because of its distinct and favorable antimicrobial susceptibility pattern, the specific identification of *N. cyriacigeorgica* may improve clinical management. Our index case suffered from a life-threatening pneumonia due to infection with *N. cyriacigeorgica*, was treated with TMP-SMX and imipenem-cilastatin, and recovered. Previously, patients with *N. cyriacigeorgica* infection have done well when treated with a variety of anti-nocardial regimens (2, 4, 11), although treatment failures have also been noted (22). In this study, we present data to indicate that *N. cyriacigeorgica* may be more susceptible to imipenem than meropenem and that amikacin and imipenem appear to work synergistically in vitro against *N. cyriacigeorgica*. However, an optimal management protocol for nocardiosis has not been defined and guidelines for specific treatment by species are needed.

*N. cyriacigeorgica* is an emerging pathogenic entity (as defined in ref. 24) that we found to be the cause of infection in seven cases at our NYC medical center, thus representing the first such cases officially reported in the USA. These strains were identified by molecular means and confirmed by phenotypic and biochemical testing. Whether previously isolated Type VI strains in the USA are truly synonymous with *N. cyriacigeorgica* remains to be definitively proven. However, by nearly all phenotypic, chemotaxonomic, and genotypic measures that we evaluated, the Type VI strain ATCC 14759T matched the clinical isolates of *N. cyriacigeorgica* tested in this study. Similarly, the strain from the index case, as well as strains 03-70718 and 05-49102 (the only others so evaluated), each displayed a Type VI antimicrobial susceptibility pattern. Therefore, the evidence supports that the species *N. cyriacigeorica* and Type VI are overlapping. This is an important point to clarify because it would mean that, at least in the Southern USA, *N. cyriacigeorgica* is the most important cause of nocardiosis (6). The fact that *N. cyriacigeorgica* has been identified in Canada (11), and now in the North-Eastern USA with this report, suggests that *N. cyriacigeorgica* might actually be distributed across all of North America, in addition to Europe and Asia (2, 4, 8, 11, 17, 18, 22, 27, 35). Clearly, *N. cyriacigeorgica* is a species that requires closer attention and clinical microbiology laboratories need to adapt laboratory protocols for its specific identification.

Molecular techniques offer several advantages over traditional phenotypic methods of *Nocardia* spp. identification and have the potential to greatly improve patient diagnosis. To date, molecular techniques have provided the most definitive means of identifying *N. cyriacigeorgica*. As we noted
significant microheterogeneity in the targets of sequencing, sequence data enabled strain
differentiation and so may also have the potential for use as a first-line screen in source
investigations of suspected nosocomial transmissions.

In this study, we also evaluated the chemotaxonomic properties of molecularly validated
isolates of *N. cyriacigeorgica*. All strains were distinguished from the *N. asteroides* laboratory
strain ATCC 19247\textsuperscript{T} and the type strains of other clinically relevant *Nocardia* spp. by a
combination of their relatively rapid growth rate, differential agar type-specific colonial
appearance, the microscopic appearance of their aerial hyphae, as well as their unique phenotypic
and biochemical test profiles. More strains need to be evaluated before firm conclusions can be
drawn, but the pattern of outcomes in this set of tests might serve as an aid to identify *N.
cyriacigeorgica* when molecular testing is not available.

Lastly, the differentiation of *N. cyriacigeorgica* from the *N. asteroides* laboratory strain ATCC
19247\textsuperscript{T} requires the evaluation of tests, such as for urease activity, which may not be considered
informative in the context of the *Nocardia*. As a consequence, many clinical isolates of *N.
cyriacigeorgica* are likely classified and reported as *N. asteroides*, which would explain why the
species has remained underdiagnosed as a cause of infection. To the contrary, our data illustrate
several genotypic, phenotypic, and biochemical differences between the species. In fact, the *N.
asteroides* sensu stricto species does not represent any of the common taxa associated with clinical
nocardiosis or drug pattern group and so its widely utilized representative strain ATCC 19247\textsuperscript{T}
serves as a poor comparator for clinical microbiology laboratories (6, 25). Perhaps a more
appropriate representative *Nocardia* laboratory strain, possibly a *N. cyriacigeorgica*, should be
designated. Indeed, the taxonomic nomenclature of the clinically important *Nocardia* is better
clarified now that the former *N. asteroides* complex has been resolved into separate species that
are clearly distinguished from the non-infectious *N. asteroides* sensu stricto species. It is hoped
that with increased awareness of *N. cyriacigeorgica* as a distinct pathogenic entity, and improved
diagnosis, an increased understanding of its biology, epidemiology, and optimal course of
treatment for infection will follow.
Sequences Submitted to GenBank

*N. cyriacigeorgica* 16S rRNA and *hsp65* sequences derived in this study were submitted to GenBank under the following accession numbers; 16S rRNA: EF127493.1, EF127494.1, EF127495.1, EF127496.1, EF127497.1, EF127498.2, EF127499.1, EF127500.1; *hsp65*: EF127503.1, EF127504.1, EF127505.1, EF127506.1, EF127509.1, EF127510.1, EF127511.1, EF127512.1.

Isolates Submitted to the American Type Culture Collection (ATCC)

One to two strains of each genotype and sequence subtype variant were submitted to the ATCC under the following accession numbers. Genotype I strain 02-50508: ATCC BAA-1517; genotype II strains 00-12607: ATCC BAA-1519 and 05-49102: ATCC BAA-1516; genotype III strain 06-51518: ATCC BAA-1518.

Note added in proof

Subsequent to the submission of our manuscript, Conville and Witebsky (9a) published on the comparative DNA-DNA hybridization results for *N. cyriacigeorgica* DSM44484<sup>T</sup> and drug pattern Type VI strain ATCC 14759<sup>T</sup>. Their data confirmed that these two strains are of the same species, consistent with the data presented in our current study.

Acknowledgments

The authors thank Dr. John M. Austin for assistance with interpretation of radiological studies, Dr. Richard Wallace and Barbara A. Brown-Elliott for initial antimicrobial testing, and Dr. Steven Spitalnik for support and encouragement.
References


TABLE 1. Patient characteristics and initial identification of *N. cyriacigeorgica* isolates, years 2000 to 2006a

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Sex</th>
<th>PMH</th>
<th>Diagnosis</th>
<th>Source</th>
<th>Treatment</th>
<th>Outcome</th>
<th>Isolate</th>
<th>Initial ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>56a</td>
<td>M</td>
<td>HTX</td>
<td>Pneumonia</td>
<td>Sputum, BAL, lung biopsy</td>
<td>SXT / IPM</td>
<td>Recovered</td>
<td>06-51518</td>
<td><em>N. cyriacigeorgica</em></td>
</tr>
<tr>
<td>59</td>
<td>M</td>
<td>AIDS</td>
<td>Respiratory distress</td>
<td>Sputum</td>
<td>Not available</td>
<td>LTFU</td>
<td>05-49182</td>
<td><em>N. asteroides</em> Type VI</td>
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<tr>
<td>27c</td>
<td>M</td>
<td>CGD</td>
<td>Lung abscess</td>
<td>Lung biopsy</td>
<td>SXT / MEM then IPM / LZD</td>
<td>Recovered</td>
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<td><em>N. asteroides</em> Type VI</td>
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<td>RA, ILD, DM</td>
<td>Pneumonia</td>
<td>Sputum</td>
<td>SXT / IPM / AMK</td>
<td>LTFU</td>
<td>03-23155</td>
<td><em>N. asteroides</em> complex</td>
</tr>
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<td>73</td>
<td>M</td>
<td>HTX, COPD</td>
<td>Pneumonia</td>
<td>Tracheal aspirate</td>
<td>SXT / TOB</td>
<td>Recovered</td>
<td>02-50508</td>
<td><em>N. asteroides</em> complex</td>
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<td>19c</td>
<td>M</td>
<td>CGD</td>
<td>Pharyngeal abscess</td>
<td>Biopsy</td>
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<td><em>Nocardia</em> sp.</td>
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<td>Sarcoidosis, DM</td>
<td>Orbital abscess</td>
<td>Biopsy</td>
<td>I&amp;D</td>
<td>LTFU</td>
<td>00-7841</td>
<td><em>Nocardia</em> sp.</td>
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a PMH, past medical history; Initial ID, original identity after molecular and/or phenotypic testing; HTX, heart transplant; CGD, chronic granulomatous disease; RA, rheumatoid arthritis; ILD, interstitial lung disease; DM, diabetes mellitus type II; COPD, chronic obstructive pulmonary disease; BAL, bronchoalveolar lavage fluid; SXT, trimethoprim-sulfamethoxazole; IPM, imipenem; MER, meropenem; LZD, linezolid; AMK, amikacin; TOB, tobramycin; I&D, incision and drainage; LTFU, lost to follow-up.

b Index patient.

c Siblings.
<table>
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<tr>
<th>Strain</th>
<th>Genotype</th>
<th>16S rRNA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>hsp65&lt;sup&gt;b&lt;/sup&gt;</th>
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<td></td>
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<td>448 1427 1480</td>
<td>216 249 279 282 285 342 345 408 435 438 453 460 546 561</td>
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<td>ATCC 14759&lt;sup&gt;T&lt;/sup&gt;</td>
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<td>T G G G</td>
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<tr>
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<tr>
<td>06-51518&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Y C G C C G C d G</td>
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<sup>a</sup> Nucleotide position numbers for 16S rRNA as for *Escherichia coli* (GenBank accession number J01859). Genotype assignments are based on SNPs in 16S rRNA positions 1427 (29) and 1480.

<sup>b</sup> Nucleotide position numbers for hsp65 as in *N. farcinica* strain IFM 10152 (GenBank accession number NC_006361).

<sup>c</sup> DSM 44484<sup>T</sup> sequences from GenBank (16S rRNA: AF430027; hsp65: AY756522).

<sup>d</sup> Non-synonymous SNP.

<sup>e</sup> Isolate from the index patient.
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<th>Strain&lt;sup&gt;b&lt;/sup&gt;</th>
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<th>ACE</th>
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<th>ARYL</th>
<th>αGLU</th>
<th>GLU</th>
<th>MAL</th>
<th>RHA</th>
<th>TRE</th>
<th>Growth at 45ºC</th>
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<sup>a</sup> ESC, esculin; URE, urease; ACE, acetamide; PYR, pyrrolidonyl arylamidase; ARYL, arylsulfatase; αGLU, α-glucosidase; GLU, glucose; MAL, maltose; RHA, rhamnose; TRE, trehalose.

<sup>b</sup> N. cyriacigeorgica patient isolates. Select additional Nocardia spp. were evaluated in parallel as controls, based upon the expected results of key biochemical tests.

<sup>c</sup> Isolate from the index patient.
Figure 1

A

B

C

Figure text
Figure 2
Figure 3

Day 2

A. BAP
B. BCYE
C. 7H11

Day 7

D. BAP
E. BCYE
F. 7H11

Day 1

I. BAP
J. BCYE

Day 7

I. BAP
J. BCYE
Figure 4

Day 7

A. BAP
B. BCYE
C. 7H11

D. E.
Figure 5
Figure 1. Radiological and histopathological findings from the index patient. A) Posterior-anterior chest X-ray (CXR) and B) chest computerized tomography (CT) show nodular opacities in the right lower lobe and dense consolidation in the left lower lobe (arrowheads) in addition to other small irregular opacities in the right upper lobe and elsewhere. The chest CT was performed 6 days, and the CXR 2 days, before the first positive sputum specimen was obtained. C) A CT guided biopsy from the left lung obtained 4 days later showed gram-positive, branching, beaded filaments and an acute inflammatory infiltrate (original magnification 400X).

Figure 2. Unrooted phylogenetic tree based on the concatenated 16S rRNA and hsp65 gene sequences (1872 bp in total). Sequences were aligned using Molecular Evolutionary Genetics Analysis (MEGA) software version 3.1 by ClustalW and a phylogenetic tree was constructed using the Neighbor-Joining method and tested for robustness by bootstrapping with 2000 replicates. * Denotes the isolate from the index patient.

Figure 3. Unique macroscopic and microscopic features distinguish N. cyriacigeorgica. Growth of N. cyriacigeorgica strain 06-51518 at 48 h on A) Columbia 5% sheep’s blood agar plates (BAP), B) buffered charcoal yeast extract (BCYE) agar, and C) Middlebrook 7H11 agar. Colonial morphology of N. cyriacigeorgica strain 06-51518 at 7 days on D) BAP, E) BCYE, and F) 7H11. G) Mega-colony of Type VI strain ATCC 14759T after 4 days of incubation on SAB agar and H) microscopic view (original magnification 100X) of the growing mega-colony edge. Gram stain of ATCC 14759T after I) 24 h growth and J) 7 days growth on SAB (original magnification 400X).

Figure 4. Unique macroscopic and microscopic features distinguish the N. asteroides laboratory type strain ATCC 19247T. Colony morphology varied with medium after 7 days of incubation at 35ºC. A) Individual colonies were shiny, honey-colored, slightly
elevated, and growing to a peak with an eroded surface texture on BAP, B) were similar but cream colored on BCYE agar, and C) were white and dome-shaped, with a buff velvety surface on 7H11 agar. D) Mega-colonies after 4 days of incubation on SAB were coral-colored, flat, and moderately ruffled. E) Microscopically, aerial hyphae protruding from these large colonies had a bundled, pointed-end appearance. Note that all *N. cyriacigeorgica* differed from the *N. asteroides* species type strain in each of the above descriptions (compare with Figure 3) as well as by urease activity and the ability to grow at 45°C (see Table 3).

**Figure 5.** Differential carbapenem susceptibility of *N. cyriacigeorgica* and synergistic activity of amikacin and imipenem. A) Antimicrobial susceptibility and synergy were evaluated for amikacin (upper right), imipenem (upper left/lower right), and meropenem (lower left) by disk diffusion. B) Minimal inhibitory concentrations (MICs) for imipenem (left) and meropenem (right) were determined by Etest. Results for Type VI strain ATCC 14759<sup>T</sup> are shown and are representative of all *N. cyriacigeorgica* clinical isolates. *N. cyriacigeorgica* strains also produced a brown pigment when grown on Mueller-Hinton agar that was clearly visible on the plate reverse (not shown). Note that, based upon preliminary testing, disks were spaced at a nonstandard 3.5 cm distance apart in order to best illustrate synergy between amikacin and imipenem.