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Species Spectrum of *Nocardia* spp. Isolated from Suspected Tuberculosis Patients*

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Abstract

Traditionally *Nocardia asteroides* was considered the predominant species of causing nocardiosis. The improved identification of isolates using molecular techniques have shown that the genus exhibits considerable taxonomic complexity and phenotypic base identification can be ambiguous. The aim of this study was to assess the species distribution of *Nocardia* strains mostly recovered from patients suspected of having tuberculosis, during three years period (2009-2012). The clinical isolates were identified to species level using conventional tests and genotypic methods using single and multi locus sequence analysis (MLSA) of 16S rRNA, *gyrB* and *secA*1 genes. Nocardiosis was diagnosed in 46 patients. The most frequent underlying condition were organ transplantation (6 patients; 13%), cancer (6 patients; 13%), human immunodeficiency virus (HIV) (6 patients; 13%), non-infectious chronic lung disease (5 patients; 10.8%) and tuberculosis (4 patients; 8.7%). *Nocardia* species was recovered from 46 different clinical specimens, the most common of which was bronchoalveolar lavage (BAL) (43.5%). Eleven different *Nocardia* species were identified: *N. asteroides* (n = 12), *N. cyriacigeorgica* (n = 9), *N. farcinica* (n = 7), *N. wallacei* (n = 6), *N. carnea* (n = 3), *N. otitidiscaviarum* (n = 3), *N. abscessus* (n = 1), *N. arthritidis* (n = 1), *N. kruczakiae* (n = 1), *N. nova* (n = 2) and *N. veterana* (n = 1). In conclusion, infection caused by *Nocardia* species appears to be more common than generally appreciated. The current study provides further evidence that *Nocardia* species are capable of causing a wide range of human diseases in healthy and immunocompromised patients. MLSA is a reliable method for accurate species identification of *Nocardia*

*The GenBank accession numbers of investigated clinical isolates of Nocardia determined in this work are as follows: KC262083-KC262128, KC262130-KC262175 and KC296657-KC296702 for the 16S rRNA, *gyrB* and *secA*1, respectively.
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isolates and would be more feasible for routine use in clinical laboratories.

Keywords

***Nocardia,* Infection, Identification, MLSA**

1. Introduction

Members of the genus *Nocardia* are aerobic, filamentous branching bacilli, gram positive and Partial acid-fast, which include more than 90 different species (http://www.bacterio.cict.fr/n/nocardia.html). The species of *Nocardia* normally are saprophytes of soil and thought to have a role in the decay of organic plant material. However, *Nocardia* have increasingly been isolated as infectious agents in immune-suppressed patients and sometimes in healthy individuals and causing localized or disseminated infections, ranging from pulmonary and wound infections to brain abscesses, and bacteremia [1]. Since the most common manifestation of nocardial infection is pneumonia, patients with fever, weight loss, cough, pleuritic chest pain, and dyspnea might be confused with other chronic lung infections such as TB or invasive fungal infection [2] [3]. While taxonomy within the genus *Nocardia* is changing rapidly as the recognition and description of new species continues, it is not easy to identify *Nocardia* isolates to species level by conventional methods [1] [4]. In Iran, where tuberculosis is prevalent and considers as one of the most challenges of public health, the role of *Nocardia* as causative agent of pulmonary and other form of infections has been neglected. Due to little information available from Iran, the aim of the present study was to clarify the species spectrum of the isolates presumptively assigned to the genus *Nocardia*, recovered from different clinical samples of patients by morphological tests. For definite and reliable identification, the clinical isolates were subjected to sequence base identification by using MLSA of 16S rRNA [5], subunit A of the SecA preprotein translocase (*secA*1) and the subunit of a type II DNA topoisomerase (*gyrB*) [6].

2. Material and Method

2.1. *Nocardia* Strains

In a three-year study conducted from 2009 to 2012, 22 and 10 different isolates of *Nocardia* recovered from patients whom were suspected to have mycobacterium related complications in Infectious and Tropical Diseases Research Center (Khuzestan, Ahvaz, Iran) and Samyar Medical Laboratory (Tehran, Iran) respectively. At the same time 14 different clinical isolates of *Nocardia* from other parts of Iran (other than Tehran and Khuzestan provinces) referred to Infectious and Tropical Diseases Research Center (Khuzestan, Ahvaz, Iran) for identification. The patients' history, which the isolates were belonged, is summarized in **Table 1**. Most of the patients with pulmonary disease diagnosed as tuberculosis cases and put on anti-tuberculosis drugs including isoniazid and rifampicin for six months, which had either not responded to treatment or had poor, responded with subsequent relapse.

2.2. Phenotypic Identification

All *Nocardia* strains from respiratory samples were recovered on Löwenstein-Jensen (LJ) medium with strains from other samples, were isolated on blood agar medium. The strains were examined for growth rate, macroscopic and microscopic morphological features, growth at different temperature and a battery of biochemical tests including growth in lysozyme, growth at 45˚C, arylsulfatase (14 days), urease activity and hydrolysis of adenine, casein, esculin, hypoxanthine, tyrosine and xanthine and utilization of asetamide as the sole carbon source according to standard procedures [7].

2.3. Molecular Identification Using 16S rRNA, *gyrB* and *secA*1 Genes Sequencing

Genomic DNA was extracted from bacterial cultures using DNA isolation kit (Qiagen, CA, USA) according to the manufacturer's instructions and stored in −20˚C until use.

Nearly full length of the 16S rRNA genes from isolates were amplified using primers pA (5'-AGAG TTTGATCCTGGCTCAG-3') and pI (5'-TGCACACAGGCCACAAGGGA-3') as described previously [5].

Table 1. Characteristics and outcome of 46 patients with Nocardiosis.

APN000	Sample source	G/A	PMH	Main symptoms	Chest X-ray	Diagnosis by clinical findings	Primary identification	Identification by	
								Phenotypic tests	MLSA
7	Leg abscess	F (66)	Healthy	Subcutaneous abscesses	ND	Mycetoma	*Nocardia* sp.	*Nocardia* sp.	*N. otitidiscaviarum*
9	BAL	F (64)	Kidney transplant recipient	Fever, cough	Irregular nodular lesions	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
10	BAL	M (59)	COPD	Fever, cough	Cavitation	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
11	Wound infection	F (62)	HIV	Fever, weight loss	NA	Mycetoma	*Streptomyces* sp.	*N. asteroids*	*N. asteroides*
12	BAL	M (34)	Chronic lymphocytic leukemia	Dyspnea, cough	Pleural effusion	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
13	Biopsy	M (19)	Soft tissue abscess	Subcutaneous nodules	ND	Mycetoma	*Nocardia* sp.	*N. asteroids*	*N. cyriacigeorgica*
14	Blood	M (44)	BMT	Fever, weight loss	ND	ND	*Nocardia* sp.	*Nocardia* sp.	*N. otitidiscaviarum*
15	BAL	F (71)	Chronic bronchitis	Dyspnea, cough	Bilateral involvement	Tb	Fungi	*N. asteroids*	*N. cyriacigeorgica*
16	Blood	F (47)	BMT	Fever, w eight loss	ND	Fungal infection	*Nocardia* sp.	*Nocardia* sp.	*N. otitidiscaviarum*
18	Sputum (3)	F (66)	COPD	Dyspnea	Consolidative with pleural	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
22	BAL	F (71)	HIV	Fever, cough	Pleural effusions	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
23	Soft tissue biopsy	M (45)	Healthy	-	-	ND	*Nocardia* sp.	*Nocardia* sp.	*N. nova*
24	Oral ulcers	M (32)	Pamphlgus	Oral ulcers	-	ND	*Streptomyces* sp.	*N. blacklockiae*	*N. wallacei*
25	Blood	F (47)	HIV	Fever	ND	ND	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
28	BAL	F (72)	Recurrent CMV, HIV	Fever, cough	Cavitation	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
29	BAL	F (71)	Healthy	Fever, cough	Infiltrates	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
30	Sputum and BAL	M (42)	HIV	Fever, general weakness and dysuria	Infiltrates	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. arthritidis*
31	BAL	F (65)	Kidney transplant recipient	Fever, cough	Diffuse pneumonic infiltrates	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
32	Brain abscess	M (32)	Brain abscess	FUO	NA	Nocardiosis	*Nocardia* sp.	*N. asteroids*	*N. cyriacigeorgica*
35	Leg discharges (2)	M (49)	DM	Swelling left leg, purulent wound	ND	Mycetoma	*Nocardia* sp.	*N. blacklockiae*	*N. wallacei*
39	BAL	F (67)	Tb	Fever, cough	Cavitation	Tb	*Nocardia* sp.	*N. asteroids*	*N. cyriacigeorgica*
40	BAL	F (31)	Healthy	Fever, cough, chest pain	Nodule	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. carnea*
41	Blood	M(66)	Mitral valve prosthesis	Post operative fatigue, chest pain	ND	ND	*Nocardia* sp.	*N. blacklockiae*	*N. wallacei*
42	BAL	F (81)	Neo	Fever, cough, chest pain	Infiltrate	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. carnea*

Continued

43	Sputum (3)	M (62)	Ischemic heart disease	Fever, cough	Small irregular nodular lesions	Tb	*Nocardia* sp.	*N. asteroids*	*N. wallacei*
47	BAL	M (67)	Chronic bronchitis	Fever, cough	Diffuse pneumonic infiltrates	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
48	BAL	M (59)	Chronic bronchitis	Fever, cough	Diffuse pneumonic infiltrates	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
49	Sputum, BAL	M (42)	Follicular non-Hodgkin	Dyspnea, cough	Bilateral involvement	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
50	Brain abscess	M (59)	Brain abscess	FUO, weight loss, headache	CT scan, cerebral abscess	Nocardiosis	Fungi	*Nocardia* sp.	*N. carnea*
51	BAL	F (47)	Healthy	Fever, chest pain, weight loss	Cavitation	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. nova*
52	BAL	F (55)	Healthy	Fever, cough, chest pain	Nodule	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
53	Biopsy	F (18)	Skin graft recipient	Weakness, soft tissue abscess	NA	Nocardiosis	*Streptomyces* sp.	*N. asteroids*	*N. wallacei*
54	Biopsy	F (28)	Soft tissue abscess	Subcutaneous nodules	ND	Mycetoma	*Streptomyces* sp.	*N. blacklockiae*	*N. wallacei*
55	BAL	F (81)	Tb	Fever, cough	Cavitation	Tb	*Nocardia* sp.	*N. asteroids*	*N. cyriacigeorgica*
56	Blood	M (32)	Liver transplant recipient	FUO	ND	ND	*Streptomyces* sp.	*N. asteroids*	*N. cyriacigeorgica*
69	Brain abscess	F (64)	Brain abscess	FUO, weight loss, headache	CT scan, cerebral abscess	Nocardiosis	Fungi	*N. asteroids*	*N. cyriacigeorgica*
71	Sputum (3)	F (64)	Tb, HIV	Fever, cough	Cavitation	Tb	*Streptomyces* sp.	*N. asteroids*	*N. asteroides*
77	Brain abscess	F (50)	Multifocal brain abscesses	FUO, headache	CT scan, cerebral abscess	Nocardiosis	*Nocardia* sp.	*N. asteroids*	*N. cyriacigeorgica*
78	Sputum	M (88)	Neo	Chest pain	Cavitation	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. veterana*
79	Bone marrow	M (14)	Hemophili	Fever, Dyspnea, cough	ND	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. abscessus*
80	BAL	F (73)	Neo	Fever, chest pain, weight loss	Infiltrate	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. kruczakiae*
1390	Sputum (4)	M (48)	Neo	Fever, cough, chest pain	Cavitation	Tb	*Nocardia* sp.	*Nocardia* sp.	*N. farcinica*
SM25	BAL	M (63)	Liver transplant recipient	Fever, cough	Small irregular nodular lesions	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
SM26	BAL	F (42)	Tb, HIV	General weakness, dysuria	Cavitation	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
SM30	BAL	F (21)	Follicular non-Hodgkin	Fever, cough	Diffuse pneumonic infiltrates	Tb	*Nocardia* sp.	*N. asteroids*	*N. asteroides*
SM62	Blood	F (46)	Kidney transplant recipient	FUO	ND	ND	*Streptomyces* sp.	*N. asteroids*	*N. cyriacigeorgica*

Abbreviations: G/A, gender/age; PMH, past medical history; F, female, ND, not determined; BAL, bronchoalveolar lavage; M, Male; COPD, chronic obstructive pulmonary disease; HIV, human immunodeficiency virus; NA, not available; BMT, bone marrow transplantation; CMV, cytomegalovirus, FUO, fever unknown origin, DM, diabetes mellitus; Tb, tuberculosis; Neo, neoplasia, CT, computed tomography.

The sequences of amplified PCR products of 16S rRNA gene for each isolate were determined by using an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems, CA, USA) following the standard protocol of the supplier.

PCR amplification and sequencing of *gyrB* and *secA*1 for each strain was carried out by the method of MacTaggart *et al*. (2010) using two sets of specific primers including Noc-*gyrB*-F (5'-CTTCGCCAACACCATCAACAC-3') and Noc-*gyrB*-R (5'-TGATGATCGACTGGACCTCG-3') for amplification of 480 bp of *gyrB* gene and *secA*1-F47 (5'-GCGACGCCGAGTGGATGG-3') and *secA*1-ConR2 (5'-TTGGCCTTGATGGCGTTGTTC-3') for amplification and sequencing of 440 bp of *secA*1 gene as described previously [6].

2.4. Analysis of Sequence Data

The obtained sequences for each isolate from different loci were aligned separately and compared with all existing relevant sequences of *Nocardia* retrieved from GenBank database by using jPhydit program [8]. Percentages of similarity between sequences of each gene were determined by comparing sequences search to an in-house database of 16S rRNA, *gyrB* and *secA*1 genes sequences.

Phylogenetic trees were obtained from DNA sequences by using the NJ method K2P distance correction model with 1000 bootstrap replications supported by the MEGA 4.1 software [9]. To better elucidate the relatedness of the clinical isolates with valid species of *Nocardia*, the respective sequences of 16S rRNA, *gyrB* and *secA*1 genes were concatenated in single, 2346 bp long, strands using the jPhydit program [8]. The sequences were then aligned and analyzed in the MEGA 4.1 beta program [9] with the NJ method bootstrapped with 1000 replicates.

3. Results

The etiologic role of the isolates might be inferred from the growing *Nocardia* isolates were microscopically observed in most of clinical specimens of each patient. Furthermore, in the case of specimens from non-sterile site such as sputum, repeated sampling yielded the same microorganisms in pure culture, which confirmed clinical significance of the isolates. Populations demographics have been inadequately considered because of many isolates were referred to our laboratory for identification, so it was impossible to establish the incidence rate of nocardiosis in our study. The individual characteristics of the 46 patients with nocardiosis are shown in **Table 1**. Nocardiosis was diagnosed in 25 (54.3%) women. Age ranged from 18 to 88 years. Most of the patients had at least one significant underlying condition. The most frequent were organ transplantation (6 patients; 13%), cancer (6 patients; 13%), HIV (6 patients; 13%), noninfectious chronic lung disease (5 patients; 10.8%) and Tuberculosis (4 patients; 8.7%). *Nocardia* species was recovered from 46 different clinical specimens, the most common of which were BAL (43.5%). The most common symptoms were fever (69.5%) and cough (47.8%). Chest X-ray findings included infiltrates in 17.4% of cases and cavitations in 19.6%. Nocardiosis was pulmonary in 27 of the 46 patients (58.7%), disseminated in 7 (15.2%) and central nervous system disease (brain abscess) in 4 (8.7%).

3.1. Species Identification by Phenotype Tests

The taxonomic status of ten clinical isolate, provisionally assigned as *Streptomyces* spp. or fungi based on macroscopic morphology but using of growth in lysozyme and modified Kinyoun staining assigned all of the isolates grouped to the genus *Nocardia*. According to growth characteristics and biochemical tests, the *Nocardia asteroids* complex like was the most common frequently encountered *Nocardia* species (45.6%) among clinical isolates (**Table 1**), but the remaining strains were identified to the genus level only.

3.2. Species Identification by Sequencing Tests

In the current study, 46 clinical strains of *Nocardia* with documented nocardiosis were diagnosed from 2009 to 2012. During the study, sequences of three genes were analyzed separately and then a combined database used to create a single alignment dataset. Comparative sequence analysis of 16S rRNA, *gyrB* and *secA*1 genes provided the basis for reliable identification of each isolates. The result of the sequence based identification was presented in **Table 2**.

Table 2. Details of identification of clinical isolates of *Nocardia* by sequence analysis.

APN-000	Percentage of Similarity (number of nucleotide differences) based on:			MLSA
	16S rRNA(~1434 bp)	*gyrB* (482 bp)	*secA*1 (432 bp)	
79	100% *N. abscessus* ATCC BAA 279^T	98.9% (5) *N. abscessus* DSM 44432^T	99.7% (5) *N. abscessus* DSM 44432^T	*N. abscessus*
10, 11, 28, 31, SM25, SM26,SM30	100% *N. asteroides* Geno I DSM43757 99.5% (8) *N. asteroides* Geno III DSM 43255 99.2% (11) *N. asteroides* Geno II DSM 43258	100% *N. asteroides* ATCC19247	100% *N. asteroides* ATCC19247	*N. asteroides*
12, 47, 48	100% *N. asteroides* Geno II DSM 43258 99.3% (10) *N. asteroids* Geno III DSM 43255 99.2% (11) *N. asteroides* Geno I DSM43757	98.9% (5) *N. asteroides* ATCC19247	97.2% (12) *N. asteroides* ATCC19247	*N. asteroides*
49, 71	100% *N. asteroides* Geno II DSM 43258	98.5% (7) *N. asteroides* ATCC19247	97.9% (9) *N. asteroides* ATCC19247	*N. asteroides*
13, 15, 32, 39, 55	100% *N. cyriacigeorgica* Geno I DSM 44484	100% *N. cyriacigeorgica* DSM 44484	100% *N. cyriacigeorgica* DSM 44484	*N. cyriacigeorgica*
SM62	99.9% (1)% *N. cyriacigeorgica* Geno I DSM 44484	95.9% (20) *N. cyriacigeorgica* DSM	97.9% (9) *N. cyriacigeorgica* DSM 44484	*N. cyriacigeorgica*
66, 77	100% *N. cyriacigeorgica* Geno I DSM 44484	100% *N. cyriacigeorgica* DSM 44484	99% (4) *N. cyriacigeorgica* DSM 44484	*N. cyriacigeorgica*
69	99.9% (1)% *N. cyriacigeorgica* Geno I DSM 44484	95.9% (20) *N. cyriacigeorgica* DSM	97.9% (9) *N. cyriacigeorgica* DSM 44484	*N. cyriacigeorgica*
9, 18	100% *N. farcinica* Geno II DSM 44562 99.9% (1) *N. farcinica* Geno I DSM 43665^T	99.4% (3) *N. farcinica* ATCC 1318	99.5% (2) *N. farcinica* ATCC 1318	*N. farcinica*
22, 25, 29, 52, 1390	100% *N. farcinica* Geno I DSM 43665^T 99.9% (1) *N. farcinica* Geno II DSM 44562	100% *N. farcinica* ATCC 1318	100% *N. farcinica* ATCC 1318	*N. farcinica*
24	100%*N. wallacei* ATCC 49873	100% *N. wallacei* ATCC 49873	100% *N. wallacei* ATCC 49873	*N. wallacei*
35	100%*N. wallacei* ATCC 49873	100% *N. wallacei* ATCC 49873	99.7% (1) *N. wallacei* ATCC 49873	*N. wallacei*
41	100%*N. wallacei* ATCC 49873	99.4 (3) *N. wallacei* ATCC 49873	99% (4) *N. wallacei* ATCC 49873	*N. wallacei*
43	100%*N. wallacei* ATCC 49873	99.2% (3) *N. wallacei* ATCC 49873	99 % (4) *N. wallacei* ATCC 49873	*N. wallacei*
53	100%*N. wallacei* ATCC 49873	99.4 (3) *N. wallacei* ATCC 49873	99.7 % (1) *N. wallacei* ATCC 49873	*N. wallacei*
54	100%*N. wallacei* ATCC 49873	99.2% (3) *N. wallacei* ATCC 49873	100% *N. wallacei* ATCC 49873	*N. wallacei*
7, 14, 16	100% *N. otitidiscaviarum* Geno I DSM 43242	100% *N. otitidiscaviarum* DSM 43242	100% *N. otitidiscaviarum* DSM 43242	*N. otitidiscaviarum*
23	99.7% (4) *N. nova* Geno I JCM 6044	99.6% (2) *N. nova* DSM 44481	99.3% (3) *N. nova* DSM 44481	*N. nova*
51	99.6% (5) *N. nova* Geno V DSM 40806	99.6% (2) *N. nova* DSM 44481	99.3% (3) *N. nova* DSM 44481	*N. nova*
80	100% *N. kruczakiae* ATCC BAA-948	100% *N. kruczakiae* ATCC BAA-948	100% *N. kruczakiae* ATCC BAA-948	*N. kruczakiae*
50	100% *N. carnea* Geno I DSM 43397	100% *N. carnea* DSM 445589 97.9% (10) *N. carnea* IFM 0237	100% *N. carnea* DSM 44558, 97.9% (9) *N. carnea* ATCC 6847	*N. carnea*
40, 42	100% *N. carnea* Geno III DSM 46071	100% *N. carnea* IFM 0237	100% *N. carnea* ATCC 6847 97.9% (9) *N. carnea* DSM 44558	*N. carnea*
30	99.9% (1) *N. arthritidis* IFM 10035^T	100% *N. arthritidis* DSM 44731	100% *N. arthritidis* DSM 44731	*N. arthritidis*
78	100% *N. veterana* DSM 44445^T	100% *N. veterana* DSM 44445^T	100% *N. veterana* DSM 44445^T	*N. veterana*

3.2.1. Analysis of 16S rRNA

The determined nucleotide sequences were compared pairwise for similarity; the results showed that the 46 *Nocardia* strains were closely related to each other and were distinct from other genera. In general, 95% to 100% similarity (interspecies divergence) was observed among isolates in compare with those of corresponding type strains. All clinical isolates of *Nocardia* were clearly differentiated and formed distinct branches in phylogenetic tree of 16S rRNA [10]. Previously reported intraspecies variation (various genotypes) based on single-nucleotide polymorphism (SNP) of the 16S rRNA gene [10]. Intraspecies divergence of 16S rRNA among clinical isolates allowed the reliable identification and definition of genotypes within tree which were supported by high bootstrap values (**Figure 1**). Clinical isolates including APN-00010, APN-00011, APN-00028, APN-00031, APN-000SM25, APN-00026, APN-000SM30, APN-00012, APN-00047, APN-00048, APN-00049, APN-00071 confidently identified by sequence analysis of 16S rRNA as *N. asteroides* (n = 12) as the most frequent species.

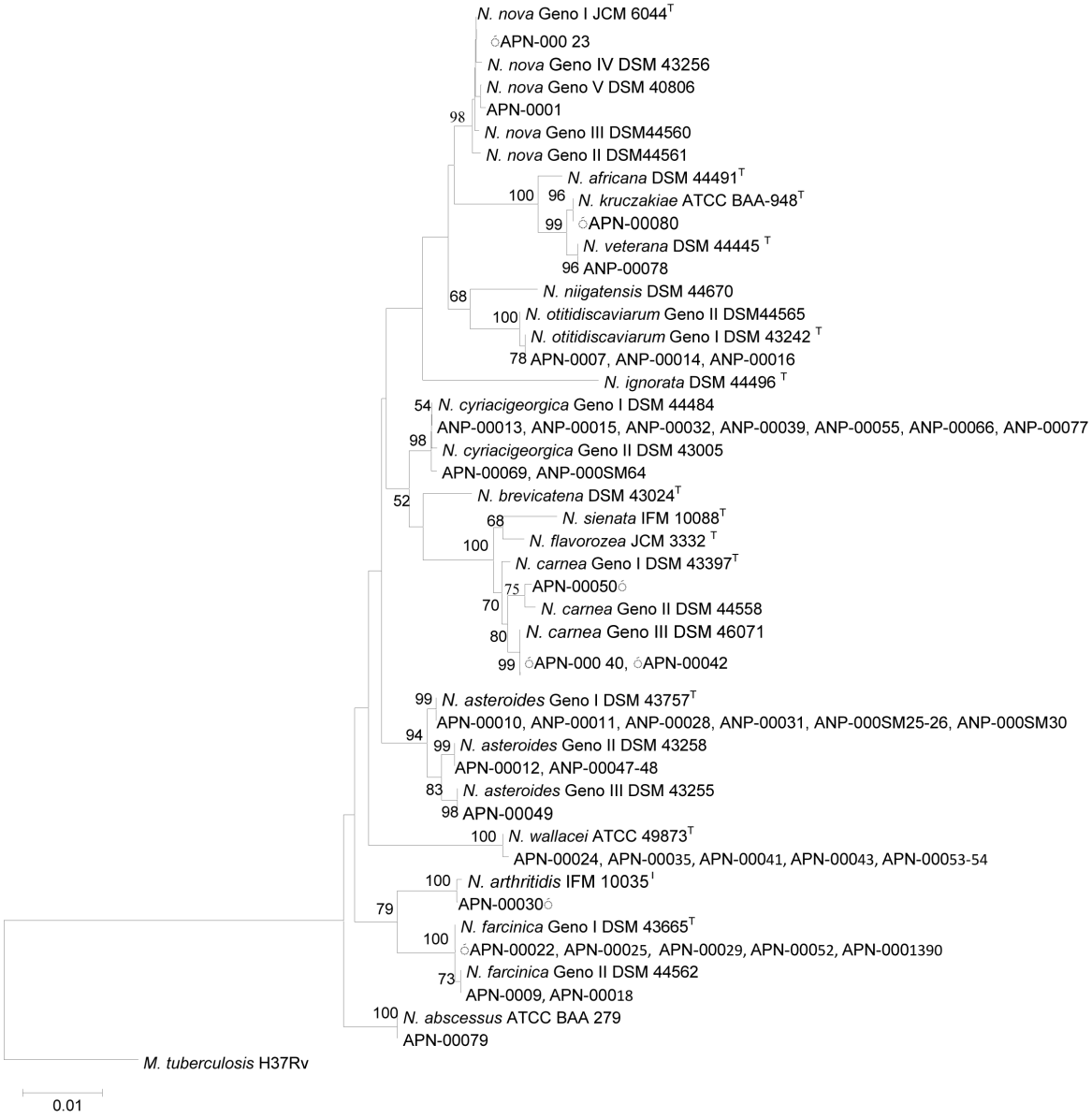

Figure 1. 16S rRNA sequence-based phylogenetic tree of clinical isolates of *Nocardia* with those of closely related species which computed by the NJ analyses and K2P model. The support of each branch, as determined from 1000 bootstrap samples, is indicated by percentages at each node. Bar 0.005 substitutions per nucleotide position.

Nucleotide sequence variations of 16S rRNA among the clinical isolates of *N. asteroides* were observed ranging from 0 to 11 bp differences (intraspecies divergence, 0.8%) (**Table 2**). The isolates including APN-00013, APN-00015, APN-00032, APN-00039, APN-00055, APN-00066, APN-00077, APN-000SM62 and APN-00069 identified as *N. cyriacigeorgica* as the second dominant species in our collection (n = 9). The range of nucleotide variation among the strains *N. cyriacigeorgica* was narrow (only 1 or 2 bp). The clinical isolate APN-000SM62 showed 1 bp and 2 bp mismatches to those of *N. cyriacigeorgica* genotype I and II and was considered as the new genotype (III) in this species (**Table 1**). The isolates APN-00022, APN-00025, APN-00029, APN-00052, APN-0001390, APN-0009 and APN-00018 characterized as *N. farcinica* (n = 7) as the third frequent species of *Nocardia* in the current study. The range of nucleotide variation among the strains *N. farcinica* was narrow (only 1 or 2 bp). Other isolates identified as *N. wallacei* (n = 6), *N. otitidiscaviarum* (n = 3), *N. carnea* (n = 3), *N. nova* (n = 2), *N. abscessus* (n = 1), *N. arthritidis* (n = 1), *N. kruczakiae* (n = 1) and *N. veterana* (n = 1) using 16S rRNA. Based on 16S rRNA, intraspecies divergence among the strains *N. carnea*, *N. nova*, *N. otitidiscaviarum* and *N. wallacei* were observed (**Table 1**).

3.2.2. Analysis of *gyrB* and *secA*1

All clinical isolates of *Nocardia* were clearly differentiated and forming distinct branches in phylogenetic tree of *gyrB* and *secA*1 (data not shown). The range of nucleotide diversity varied for each of the loci: 0.0 to 100 bp for *gyrB* and 0.0 to 75 bp for *secA*1. The clinical isolates of *N. asteroids*, *N. cyriacigeorgica*, *N. carnea*, *N. farcinica* and *N. nova* were divided into different genotypes which also supported by 16S rRNA analysis. In the case of isolates belongs to *N. wallacei* significant discrepancy was detected between 16S rRNA genotyping and those of observed using *gyrB* and *secA*1 (**Table 1**).

3.2.3. MLSA Based Identification

Among all 46 strains, the diversity of the concatenated 16S rRNA-*gyrB*-*secA*1 sequences ranged from 0 to 246 nucleotide differences occurring between strains. The NJ tree of the concatenated sequences (**Figure 2**) clearly showed clinical isolates clusters with bootstrap values of 99% - 100% with that of corresponding type strain. For all of the strains, species identification was assigned because their MLSA species cluster contained a single type strain. Beyond delineating species clusters, the overall structure of the NJ tree obtained from concatenated sequences of 16S rRNA-*gyrB*-*secA*1 correlated well with those of obtained by any of each locus.

4. Discussion

Because of the frequent reports and significance of *Nocardia* infections, there is an increasing need for rapid characterization of clinically isolated *Nocaradia* especially in clinical setting with high tuberculosis rate. Beyond Gram and modified-acid-fast staining, species identification of *Nocardia* relies on morphological and conventional biochemical reactions, which are cumbersome and not definitive [1] [10]. Although 16S rRNA gene sequencing, remains first choice for bacterial identification and different molecular targets represent promising alternatives [1] [11], however the reliability of species delineation on the basis of the sequence of a single gene suffers from different factors such as stochastic genetic variation, horizontal gene transfer and recombination [12]. To address this problem, MLSA has been provided very promising results for prokaryotic taxonomy and species identification [12] [13]. Recently, McTaggart *et al*. (2010) have been suggested that five-locus (16S rRNA, *gyrB*, *secA*1, *hsp65*, and *rpoB*) MLSA is a reliable method of elucidating taxonomic data however, three-locus (16S rRNA-*gyrB*-*secA*1) MLSA scheme is nearly as reliable and correctly identifying 98.5% isolates and would be more feasible for routine use in a clinical reference microbiology laboratory for species identification of *Nocardia* [6]. In the current study, 46 clinical isolates of *Nocardia* were subjected to identification using three-locus (16S rRNA-*gyrB*-*secA*1) MLSA scheme. Different genotypes of *N. asteroids*, *N. carnea*, *N. cyriacigeorgica*, *N. farcinica*, *N. otitidiscaviarum* and *N. nova* have been reported based on the 16S rRNA gene [10]. Interspecies micro-heterogeneity was also detected based upon SNP analysis of the *gyrB* and *secA*1 genes, which were relatively more polymorphic than the 16S rRNA sequences (**Table 2**). However, the importance of interspecies variation among the isolates from clinical point of view remained to be answered due to limited number of strains. The association between nocardiosis and underlying diseases has been well-documented [4] [14]-[16]. In our study, most of the cases had associated underlying diseases and corticosteroid therapy. Current study like other reports revealed that pulmonary and disseminated nocardial disease is observed mostly in immune-compromised patients, whereas primary cutaneous nocardiosis is usually an infection of immune-compe-

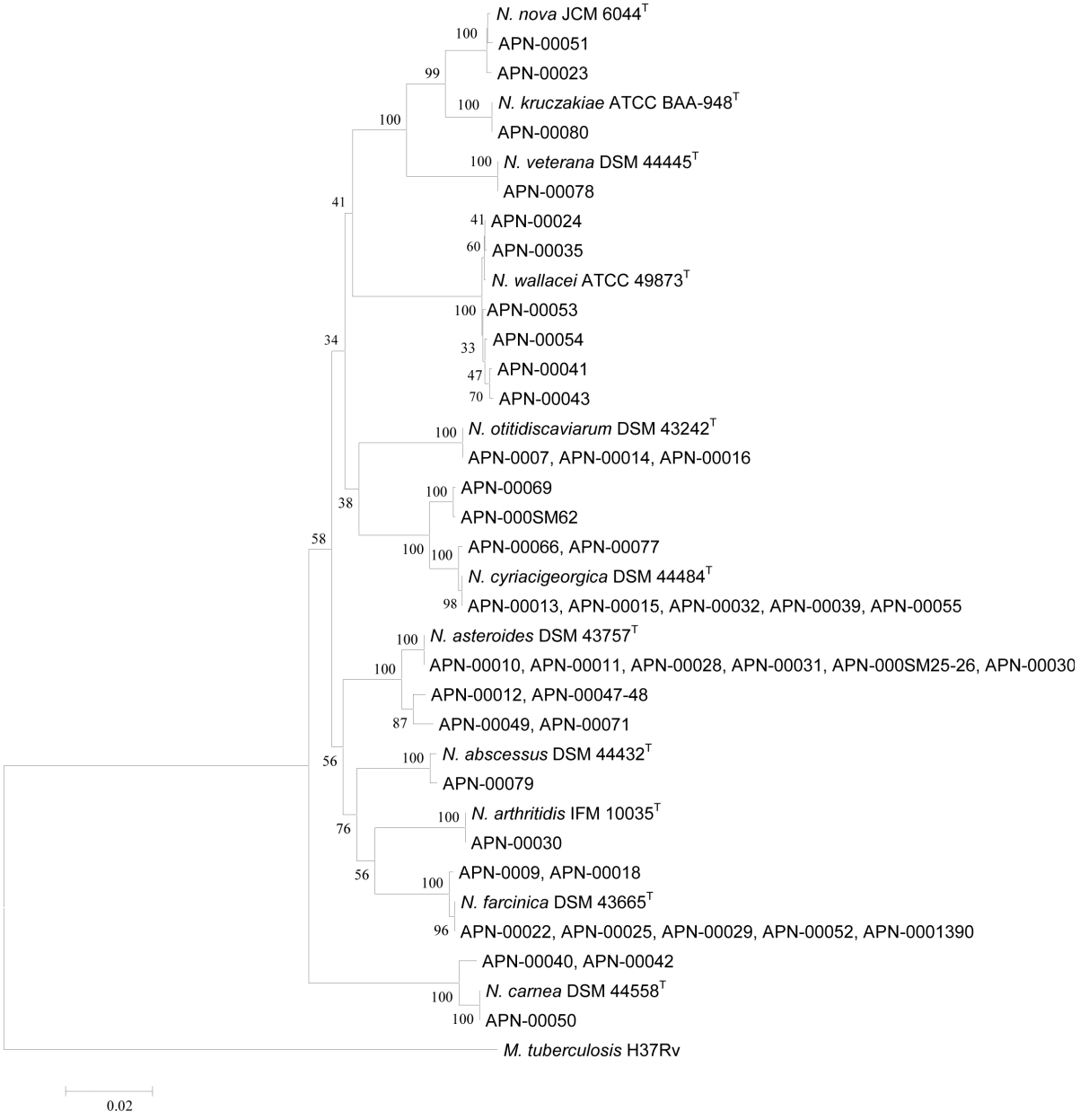

Figure 2. Phylogenetic tree of the genus *Nocardia* computed from the concatenation of 16S rRNA, *gyrB* and *secA*1 sequences by the NJ method and K2P model as the substitution model. Percentages at nodes represent bootstrap levels supported by 1000 resampled datasets. Bar 0.01 substitutions per nucleotide position.

tent hosts [16]-[19]. In recent years, nocardiosis has been increasingly reported in Iran [20]-[23], however in most of them identification of the clinical isolates were determined on the basis of microscopic examination, culturing and sometimes phenotypic tests. Twelve of the isolates (26%] were identified as *N. asteroids* as the most frequent encounter species in clinical samples by molecular methods. To best of our knowledge, this is the first report of identification of *N. asteroids* by microbiological methods from Iran as frequently encountered species in clinical samples. During past years, several cases of nocardiosis by *N. asteroids* from localized to disseminated disease, have been reported from other parts of the world [14]-[16] [24]. The second most dominant species in our clinical isolates was *N. cyriacigeorgica* (9 isolates, 19.5%). Infection due to this species was previously reported from Iran based on phenotypic methods and 16S rRNA sequencing [23]. Disease due to this species has recently been reported [25] and it represents 10.2% and 18% of the *Nocardia* isolates in Japan and

Thailand [26] and USA [27] respectively and 22% and 32.5% from two different studies from Spain [16] [24]. Together our finding from present study and several cases of nocardiosis by *N. cyriacigeorgica* from other parts of the world confirm major role of the latter species in the clinical settings. The isolation of *N. farcinica* is highly variable among countries. In current study, seven isolates identified as *N. farcinica* as first report of infections caused by this species from Iran. It has been reported to constitute 23.8%, 26.7%, 35.4% 44%, 60.3% and 33.3% and 21.6% of *Nocardia* isolates in France [14], Japan [28], Thailand [29], Belgium [30] [31], Germany [32] and Spain [16] respectively. Six of the clinical isolates were identified as *N. wallacae* as the first report of infections caused by the later species in Iran. Few cases of nocardiosis by *N. wallacae* have been reported [29]. To best of our knowledge, this is the first study to report of large number of clinical isolates of *N. wallacae*. Our data provide evidence that *N. wallacae* capable of establishing infection in immune-compromised humans. One isolate from bone marrow biopsy of a patient suffering from hemophilia syndrome identified as *N. abscessus*. Infections by *N. abscessus* have been reported from different regions such as Japan [33], Germany [34], Argentine [35] and Spain [16] [24]. Three isolates were identified as *N. otitidiscaviarum*. Importantly, two isolates recovered from blood samples of two bone marrow recipients and one from subcutaneous abscesses of healthy patient. Thus, clinicians should be aware that *N. otitidiscaviarum* tends to infect both immune-compromised and immune-competent individuals. This study is the first to report the isolation of *N. arthritidis*, *N. carnea*, *N. kruczakiae*, *N. nova* and *N. veterana* in Iran. Our data provides more evidences that *N. arthritidis*, *N. kruczakiae* and *N. veterana* as rare pathogens capable of establishing infections in concordant with previous reports [26] [36] [37].

5. Conclusion

In conclusion, *N. asteroides* was the most common pathogen causing nocardiosis in Iran, followed by *N.* cyriacigeorgica, *N. farcinica* and *N. wallacei*. In addition, patients with nocardiosis caused by rare species, including *N. abscessus*, *N. arthritidis*, *N. carnea*, *N. kruczakiae*, *N. nova*, *N. otitidiscaviarum* and *N. veterana* were also found. This nationwide study found that nocardiosis can be caused by different species of *Nocardia* and laboratory algorithms based on phenotypic identification of *Nocardia* strains unable to assign the isolates to species level. Precise and reliable identification of *Nocardia* to the species level currently requires sequence based methods and preferably MLSA of 16S rRNA, *gyrB* and *secA*1.

Conflict of Interest

None to declare.

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Abbreviations

MLSA, multi locus sequence analysis;
TB, tuberculosis;
*secA*1, gene encoding subunit A of the SecA pre protein translocase;
gyrB, gene encoding subunit of a type II DNA topoisomerase gene;
NJ, Neighbour-Joining;
K2P, Kimura's two parameter;
*hsp*65, gene encoding heat shock protein 65 KDa;
rpoB, gene encoding beta subunit RNA polymerase B.