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ORIGINAL PAPER

Comparison of restriction enzyme pattern analysis and full gene sequencing of 16S rRNA gene for *Nocardia* species identification, the first report of *Nocardia transvalensis* isolated of sputum from Iran, and review of the literature

Mehdi Fatahi-Bafghi · Parvin Heidarieh · Masoumeh Rasouli-nasab · Shadi Habibnia · Abdorazagh Hashemi-Shahraki · Seyyed Saeed Eshraghi

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Abstract Nocardial infections occur in different organs of the body and are common in immune disorder diseases of individuals. The aim of this study was to assess *Nocardia* species identification by phenotypic tests and molecular techniques applied to nocardiosis in Iranian patients. In the current study, various clinical samples were collected and cultured on conventional media and using the paraffin baiting method. Various phenotypic tests were performed. For accurate identification at the species level, restriction fragment length polymorphisms (RFLP) in the *hsp65* and partial 16S rRNA genes and full gene sequencing of the 16S rRNA gene were used. Twenty-seven *Nocardia* spp. were isolated and analysis of phenotypic tests results showed *Nocardia asteroides* complex, *Nocardia otitidiscaviarum*, *Nocardia nova*, and *Nocardia* spp. New RFLP patterns of *Nocardia* strains with *hsp65* and partial 16S rRNA genes were obtained. Full gene sequencing of the 16S rRNA gene identified *Nocardia cyriacigeorgica*, *N. otitidiscaviarum*, *Nocardia farcinica*, *Nocardia transvalensis*, and *N. nova*. *Nocardia* infections are rarely reported and this genus is the cause of various illnesses. Accurate identification of *Nocardia* spp. is important for epidemiology studies and treatment. It should also be noted that some species may have similar RFLP patterns; therefore, full gene sequencing of the 16S rRNA gene is necessary for confirmation.

Keywords Phenotypic test · *Nocardia* · *hsp65* gene · 16S rRNA gene · RFLP · Sequencing

M. Fatahi-Bafghi · M. Rasouli-nasab · S. Habibnia · S. S. Eshraghi (✉)
Department of Microbiology, Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
e-mail: eshraghs@tums.ac.ir

M. Fatahi-Bafghi
Department of Microbiology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran

P. Heidarieh
Department of Bacteriology and Virology, Alborz University of Medical Sciences, Karaj, Iran

A. Hashemi-Shahraki
Department of Epidemiology, Pasteur Institute of Iran, Tehran, Iran

Introduction

Nocardia species are Gram-positive, partially acid-fast, non-motile, aerobic actinomycetes with branched bacilli, first described and the type was isolated by Edmond Nocard in 1888 (Brown-Elliot et al. 2006). These bacterium are not part of the normal human bacterial flora (Kahn et al. 1981; Ambrosioni et al. 2010). The members of the genus *Nocardia* have GC-rich genomes and are the cause of opportunistic infections in different organs of the body, such as lung (pulmonary nocardiosis), skin (cutaneous nocardiosis), and brain abscess. *Nocardia* species are found around the world and in the environment,

including dust, water and decaying plant materials (Bafghi et al. 2014; Brown-Elliott et al. 2006; Shimizu et al. 1998; Kofteridis et al. 2005; Lai et al. 2011; Watson et al. 2011). To date, nearly 100 species of this genus have been reported (http://www.bacterio.cict.fr/n/nocardia.html) and accurate identification of these species using biochemical methods is difficult (Liu et al. 2011a; Takeda et al. 2010; Conville and Witebsky 2005). *Nocardia* infections occur in immunosuppressive, immunocompetent, and immunocompromised individuals (Dodiuk-Gad et al. 2010; Kofteridis et al. 2005). Isolation and identification of *Nocardia* spp. are necessary from clinical samples for the distinction of nocardiosis and require a skillful microbiologist (Ambrosioni et al. 2010; Inamadar and Palit 2003). The antimicrobial susceptibility profiles and treatments are different among species of *Nocardia*; therefore, accurate identification is important at the species level (Wada et al. 2003; Lai et al. 2011; Noh et al. 2011; Liu et al. 2011b). Diversity in biochemical characteristics for each *Nocardia* species can be found in the literatures. To date, the number of new species is rising and this method is time consuming and unsuitable for accurate identification at the species level. Moreover, a skilful specialist is needed to perform and interpret the analyses (Roth et al. 2003). For accurate identification, a combination of biochemical characteristics and molecular methods should be used (Isik and Goodfellow 2010; Brown-Elliott et al. 2006). From the 1990s onward, various molecular techniques have been reported for accurate identification of members of the genus *Nocardia* at the species level, including polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) and PCR-sequencing of the 16S rRNA (full gene sequencing), *rpoB*, *secA* and *gyrB* genes (Conville and Witebsky 2005; Brown-Elliott et al. 2006; McTaggart et al. 2010). The aim of this study was to determine the prevalence of *Nocardia* species in various clinical samples from Iranian patients with biochemical tests and molecular methods (PCR-RFLP of partial 16S rRNA and *hsp*65 genes and full gene sequencing of the 16S rRNA gene). The current study is the first report of cutaneous nocardiosis from Iranian patients.

Materials and methods

Isolation and phenotypic tests

Clinical samples were collected from February 28, 2008 to March 25, 2015. In the current study, 769 various clinical samples, such as sputum, BAL (bronchoalveolar lavage), cutaneous abscess, wound, brain abscess, dental abscess, CSF (cerebrospinal fluid), gastric lavage, and bone marrow biopsy, were examined. Specimens were cultured on blood agar, nutrient agar, and Sabouraud Dextrose agar with cyclohexamide using the paraffin baiting technique and were incubated at 35 °C. Colonies resembling the genus *Nocardia* were Kinyoun acid-fast, partially acid-fast, and Gram stained. Colony morphology was evaluated by stereomicroscopy. Various biochemical tests were used, including growth in lysozyme broth (Sigma-Aldrich); decomposition of L-tyrosine (Sigma-Aldrich), hypoxanthine (Sigma-Aldrich), casein (Merck- Germany), and xanthine (Sigma-Aldrich), hydrolysis of urea (Merck-Germany), esculin (Merck- Germany), and gelatin (Sigma-Aldrich); production of nitrate reductase (Sigma-Aldrich); citrate utilization (Merck- Germany); acid production of sorbitol, rhamnose, glucose, L-arabinose, D-xylose, galactose, mannitol, lactose, maltose, sucrose, raffinose, and salisin (Merck-Germany); and growth at 45 °C (Brown-Elliott et al. 2006; Workman et al. 1998; Wauters et al. 2005; Goodfellow 1973b; Goodfellow et al. 1974; Habibnia et al. 2015).

DNA extraction

Chromosomal DNA was extracted using a method reported by Bafghi et al. In brief, a small loopful of a pure culture was inoculated in 3 mL brain heart infusion (BHI) broth and incubated at 35 °C with shaking. After 5–7 days, tubes containing BHI broth cultures were centrifuged and rinsed with saline solution. Sediment was suspended in 200 µL STET (sodium chloride, Tris, EDTA, Triton X-100) buffer and boiled for 30 min. The suspension was centrifuged and the supernatant transferred to another sterile microtube. Cold 95 % ethanol was added and the tube remained at −20 °C for 60 min. After this stage, the microtube was centrifuged and the supernatant discarded. Then, 50 µL distilled water was added and stored at −20 °C for molecular analysis (Bafghi et al. 2014a).

hsp65 gene amplification for molecular identification

Primers for the 65-kDa heat shock protein gene: TB11: 5′-ACCAACGATGGTGTGTCCAT-3′ and TB12: 5′-

CTTGTCGAACCGCATACCCT-3′ (described by Telenti et al. 1993) were used to amplify a 441-bp fragment by PCR (Telenti et al. 1993). The PCR mixtures contained 3 μL of DNA from each of the isolates, 2 μL of each primer (20 pmol), 25 μL of master mix (Ampliqon, Denmark), and 20 μL sterile distilled water in a final volume of 50 μL. Amplification cycles for this gene were performed as described previously by Conville et al. including initial denaturation step: 5 min at 94 °C, 45 amplification cycles (denaturation: 94 °C for 60 s, annealing: 55 °C for 60 s, extension: 72 °C for 60 s), and a final extension step of 10 min at 72 °C. *Mycobacterium tuberculosis* H37RV was used as positive control.

Partial 16S rRNA gene amplification for molecular identification

Primers for the partial 16S rRNA gene that were used in this study were as follows:

f: 5′-CGAACGCTGGCGGCGTGCTTAAC-3′ and 16S rRNA.

r1: 5′-CCTGTACACCGACCACAAGGGGG-3′ and 16S rRNA.

r2: 5′-ACCTGTACACCAACCACAAGGGGG-3′ (targeting a 999-bp fragment, described by Conville et al.) (Conville et al. 2000). PCR mixtures contained 3 μL of DNA from each of the isolates, 0.25 μL of each primer (10 pmol), 20 μL of master mix (Ampliqon, Denmark), and 26.25 μL sterile distilled water in a final volume of 50 μL. Amplification cycles of this gene was performed as described previously by Conville et al. and included an initial denaturation step: 5 min at 94 °C, 40 amplification cycles (denaturation: 94 °C for 60 s, annealing: 68 °C for 45 s, extension: 72 °C for 60 s), and a final extension step of 10 min at 72 °C. *Nocardia asteroides* (NCBI Accession number: KP137521) was used as a positive control.

RFLP analysis

PCR amplification products of the *hsp65* and partial 16S rRNA genes were subjected to digestion using *Msp*I, *Hin*fI (Fermentas), *Bsa*HI, *Bst*EII, *Hin*PII, *Dpn*II, and *Sph*I (New England Biolabs) (Rodríguez-Nava et al. 2006; Conville et al. 2000). Ten μL of digestion reactions were electrophoresed through 3 % agarose gel (Invitrogen, USA) for 5 h (approximately 50 V/cm) and the agarose gel was stained with ethidium bromide. A 50-bp ladder (Geneon Company-Germany was used as a DNA size marker.

DNA sequence determination

Universal 16S rRNA gene primers 27f (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1525r (5′-AAGGAG GTGWTCCARCC-3′) were used for full gene sequencing of the 16S rRNA gene amplification (Chun and goodfellow 1995) and PCR sequencing was performed by the Bioneer company (South Korea). Sequences were analyzed with jPhydit software and the phylogenetic tree was drawn with MEGA5 software (Tamura et al. 2011).

Results

Culture and biochemical characteristics

Twenty-seven *Nocardia* strains were isolated from 769 patients, including those with COPD (chronic obstructive pulmonary disease) and leukemia, transplant recipients, sputum of patients with suspected tuberculosis, sputum of patients with cystic fibrosis, patients with diabetes, Pemphigus and Behçet's disease patients who used immunosuppressive drugs or corticosteroids, and healthy individuals. In the current study, there were no HIV-positive patients. The characteristics of nocardiosis-positive patients are given in Table 1. The strains were isolated from sputum and BAL (21 strains), cutaneous abscesses such as thigh abscess, breast abscess, muscle abscess, and wound (4 strains), and brain abscess (2 strains). The results of colony staining were Gram positive, partially acid-fast branching, and negative for Kinyoun acid-fast. All of the isolates grew in lysozyme broth and were negative for decomposition of L-tyrosine and casein. Four and two isolates were positive for hypoxanthine and xanthine, respectively, and one isolate was negative for urea. All isolates had aerial hypha. Phenotypic test results are shown in Table 1. Analysis of phenotypic identified showed *N. asteroides* complex, *N. otitidiscaviarum*, *N. nova*, and *Nocardia* spp.

Table 1 Phenotypic characteristics of *Nocardia* isolates

Conventional tests	M1	M2	M3	M4	M5	M6	M10	M11	M12	M13	M14	M15	M16	M17	M18
Gender	M	M	M	F	M	M	M	M	M	M	F	M	M	F	M
Age	49	58	67	72	50	45	39	52	76	50	52	54	70	50	35
Specimens	S	S	S	S	S	S	TA	S	B	B	B	B	B	BA	MA
Colony color	W	w	w	w	w	w	c	w	w	w	w	w	w	o	c
Gram-stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Partially acid fast	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Kinyoun acid-fast	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Grow at lysozyme broth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Production of Nitrate reductas	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Hydrolysis of															
Urea	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Gelatin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Casein		−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Tyrosine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Hypoxanthine	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+
Xanthine	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W
Esculin	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Growth at 45 °C	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
Utilization of															
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−
Lactose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-arabinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Maltose	−	−	−	−	−	+	−	−	−	−	−	−	−	+	−
Galactose	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

Conventional tests	M19	M20	M21	M22	M23	M24	M25	M26	M27	M28	M29	M30
Gender	F	F	M	M	F	F	M	M	F	M	M	F
Age	75	55	63	39	44	33	48	43	36	41	55	45
Specimens	S	S	S	B	B	BR	W	S	B	B	BA	S
Colony color	w	w	w	w	w	w	w	w	w	w	c	w
Gram-stain	+	+	+	+	+	+	+	+	+	+	+	+
Partially acid fast	+	+	+	+	+	+	+	+	+	+	+	+
Kinyoun acid-fast	−	−	−	−	−	−	−	−	−	−	−	−
Grow at lysozyme broth	+	+	+	+	+	+	+	+	+	+	+	+
Production of Nitrate reductas	+	+	+	−	+	+	+	+	+	+	+	+
Hydrolysis of												
Urea	+	+	+	W	+	+	+	+	+	+	+	+
Gelatin	−	−	−	−	−	−	−	−	−	−	−	−

Table 1 continued

Conventional tests	M19	M20	M21	M22	M23	M24	M25	M26	M27	M28	M29	M30
Casein	−	−	−	−	−	−	−	−	−	−	−	−
L-Tyrosine	−	−	−	−	−	−	−	−	−	−	−	−
Hypoxanthine	−	−	−	+	+	−	−	−	−	−	−	−
Xanthine	−	−	−	−	W	−	−	−	−	−	−	−
Esculin	+	+	+	+	+	+	−	+	+	+	+	+
Growth at 45 °C	+	+	+	−	−	+	−	W	+	+	+	+
Utilization of												
Citrate	+	+	−	−	+	+	−	+	+	+	+	+
L-Rhamnose	−	−	−	−	−	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−
D-Xylose	−	−	−	−	−	−	−	+	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−
Sucrose	−	−	−	−	−	−	−	−	−	−	−	−
Lactose	−	+	−	−	−	−	−	−	−	−	−	−
L-Arabinose	−	−	−	−	−	−	−	−	−	−	−	−
Glucose	−	−	−	+	−	−	−	−	−	−	−	−
Maltose	−	−	−	−	−	−	−	−	−	−	−	−
Galactose	−	−	−	−	−	−	−	−	−	−	−	−
Salicin	−	−	−	−	−	−	−	−	−	−	−	−

M Male, *F* Female, *S* sputum, *TA* thigh abscess, *B* bal, *BA* brain abscess, *MA* muscle abscess, *BR* breast abscess, *W* wound, *w* white, *c* cream, *o* orange, *W* weak reaction

Molecular methods

HSP gene RFLP

All isolates were positive for the *hsp65* gene and 65-kDa heat shock protein gene-RFLP method patterns identified *N. cyriacigeorgica* (16 isolates), *N. otitidiscaviarum* (4 isolates), *N. farcinica* (2 isolates), and *Nocardia* spp. (5 isolates). Characteristics of the RFLP patterns are shown in Table 2. Five isolates displayed seven new patterns of RFLPs that are not reported the literature and these isolates were not identified by this method (Table 2). Analysis of full gene sequencing of the 16S rRNA gene showed that M17, M18, M21, M22, and M25, which had new RFLP patterns for *hsp65*, were identified as *N. otitidiscaviarum*, *N. otitidiscaviarum*, *N. cyriacigeorgica*, *N. transvalensis* and *N. nova,* respectively.

Partial 16S rRNA gene RFLP

All isolates were positive for the partial 16S rRNA gene and the RFLP method identified *N. cyriacigeorgica* (16 isolates), *N. otitidiscaviarum* (5 isolates), *N. farcinica* (2 isolates), *N. transvalensis* (1 isolate), and *Nocardia* spp. (3 isolates). Characteristics of the RFLP patterns are shown in Table 3. One isolate (M25) displayed a new pattern that has not been reported in the literature and was not identified by this method. Two isolates (M17 and M21) had unknown partial 16S rRNA gene patterns (Table 3). Analysis of full gene sequencing of the 16S rRNA gene identified *N. otitidiscaviarum* (M17), *N. cyriacigeorgica* (M21) and *N. nova* (M25).

Full gene sequencing of the 16S rRNA gene

Phylogenetic tree of the 16S rRNA gene sequences of the isolates was constructed using the neighbor-joining algorithm with bootstrap analysis for 1000 replicates in the MEGA5 software. *N. cyriacigeorgica* (17 isolates), *N. otitidiscaviarum* (6 isolates), *N. farcinica* (2 isolates), *N. transvalensis* (1 isolate), and *N. nova* (1 isolate) were identified by this method (Table 4; Fig. 1). Alignment of selected stretches of the16S rRNA gene of all strains is shown in Tables 5, 6, 7, 8 and 9.

Table 2 Restriction patterns and identification of *Nocardia* species by PRA-*hsp*65

Isolates	PRA				
	*Bst*EII patterns	*Msp*I patterns	*Bsa*HI patterns	*Hin*fI patterns	Best matches by PRA
M1, M2, M3, M5, M6, M11, M12, M13, M14, M15, M20, M24, M26, M27, M28, M30	440	115–120/130–145/180	65/75/270–300	440	*N. cyriacigeorgica*
M10, M29	440	440	65/75/270–300	440	*N. farcinica*
M4, M16, M19, M23	440	115–120/130–145/180	60/70/305	125/315	*N. otitidiscaviarum*
M17	440	60/80/120/180	80/100/260[a]	60/150/230	Not identified
M18	440	115–120/130–145/180	35/65/150/190	125/315[a]	Not identified
M21	300/140[a]	70/110–115/145	45/75/320[a]	190/260	Not identified
M22	440	180/260[a]	40/90/200[a]	190/260	Not identified
M25	440	180/260	30/70/340[a]	140/300	Not identified

PCR-restriction enzyme analysis

[a] New patterns of our isolates

Table 3 Restriction patterns and identification of *Nocardia* species by PRA-16S rRNA gene

Isolates	PRA				
	*Sph*I patterns	*BstE*II patterns	*Dpn*II patterns	*HinP*II patterns	Best matches by PRA
M1, M2, M3, M5, M6, M11, M12, M13, M14, M15, M20, M24, M26, M27, M28, M30	1000	1000	95/200/250/455	225/350/420	*N. cyriacigeorgica*
M10, M29	1000	1000	95/200/705	55/125/175/225/420	*N. farcinica*
M4, M16, M18, M19, M23	1000	1000	95/200/250/455	75/150/350/420	*N. otitidiscaviarum*
M17	1000	Unknown pattern	95/200/250/455	75/150/350/420	Not identified
M21	Unknown pattern	270/730	1000	225/350/420	Not identified
M22	1000	270/730	95/200/700	225/350/420	*N. transvalensis*
M25	1000	270/730[a]	60/95/200/640	225/350/420	Not identified

[a] New patterns of our isolates

Table 4 Comparison of the results of phenotypic, PCR–RFLP and full gene sequencing in this study

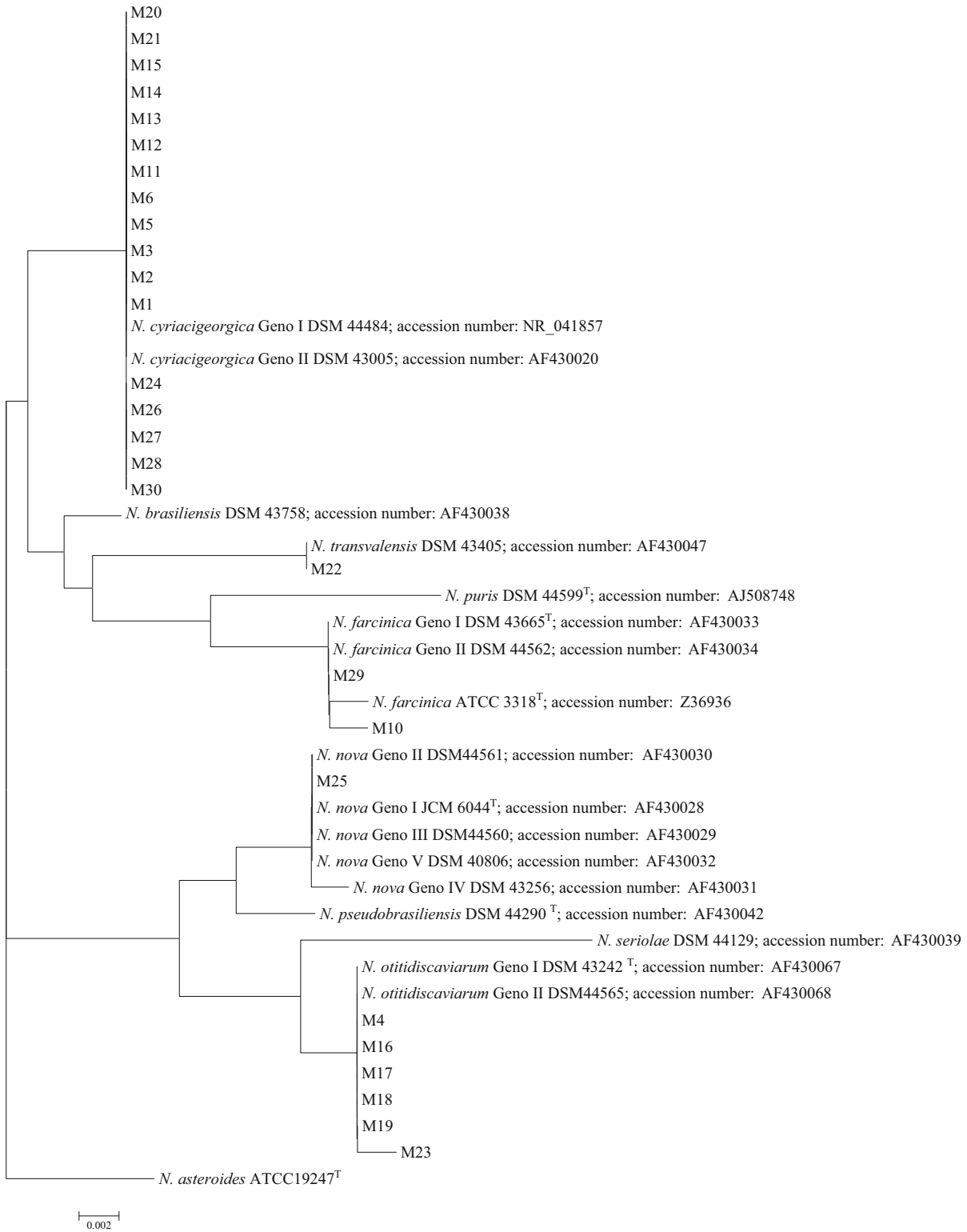
Isolates number	Species by phenotypic tests	Species by *hsp65*-RFLP	Species by 16S rRNA-RFLP	Species by sequencing (closest matches)	% Similarity/ accession number
M1	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.57/KU356884
M2	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.64/KU356885
M3	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.72/KU356886
M4	*N. asteroides* complex	*N. otitidiscaviarum*	*N. otitidiscaviarum*	*N. otitidiscaviarum*	99.79/KU356878
M5	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.83/KU356887
M6	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356888
M10	*N. asteroides* complex	*N. farcinica*	*N. farcinica*	*N. farcinica*	99.86/KU356873
M11	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.72/KU356889
M12	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356890

Table 4 continued

Isolates number	Species by phenotypic tests	Species by *hsp65*-RFLP	Species by 16S rRNA-RFLP	Species by sequencing (closest matches)	% Similarity/ accession number
M13	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/JX121854
M14	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/JX121853
M15	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/JX121852
M16	*N. asteroides* complex	*N. otitidiscaviarum*	*N. otitidiscaviarum*	*N. otitidiscaviarum*	100/KU356879
M17	*Nocardia* spp.	Not identified	Not identified	*N. otitidiscaviarum*	99.72/KU356876
M18	*N. otitidiscaviarum*	Not identified	*N. otitidiscaviarum*	*N. otitidiscaviarum*	100/KU356877
M19	*N. asteroides* complex	*N. otitidiscaviarum*	*N. otitidiscaviarum*	*N. otitidiscaviarum*	100/KU356880
M20	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356891
M21	*N. asteroides* complex	Not identified	Not identified	*N. cyriacigeorgica*	99.86/KU356892
M22	*Nocardia* spp.	Not identified	*N. transvalensis*	*N. transvalensis*	100/KU356875
M23	*N. otitidiscaviarum*	*N. otitidiscaviarum*	*N. otitidiscaviarum*	*N. otitidiscaviarum*	99.86/KU356881
M24	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.72/KU356883
M25	*N. nova*	Not identified	Not identified	*N. nova*	99.93/KU356872
M26	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356890
M27	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	99.44/KU356893
M28	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356882
M29	*N. asteroides* complex	*N. farcinica*	*N. farcinica*	*N. farcinica*	99.91/KU356874
M30	*N. asteroides* complex	*N. cyriacigeorgica*	*N. cyriacigeorgica*	*N. cyriacigeorgica*	100/KU356894

Discussion

Accurate identification of the members of the genus *Nocardia* at the species level is necessary for antimicrobial susceptibility profile prediction, treatment, and epidemiology studies (Bafghi et al. 2014b; Rodríguez-Nava et al. 2006; Kiska et al. 2002; Ambaye et al. 1997). Biochemical tests such as decomposition of L-tyrosine, hypoxanthine, casein, and xanthine and hydrolysis of gelatin give negative results in the *N. asteroides* complex. Decomposition of hypoxanthine and xanthine, hydrolysis of urea and escholin, and production of nitrate reductase are positive tests for *N. otitidiscaviarum* (Brown-Elliott et al. 2006). Previous studies have shown that PCR-RFLP of *hsp65* and 16S rRNA genes is useful for identification at the species level of the genus *Nocardia* (Conville et al. 2000; Steingrube et al.1995b, 1997; Rodríguez-Nava et al. 2006). This method (PCR-RFLP) has been demonstrated to be sensitive, less labor-intensive, and less time-consuming than traditional phenotypic methods (Steingrube et al. 1995a). Steingrube et al. in 1995, introduced patterns of *N. nova* with RFLP analysis of *hsp65* (use of *Msp*I and *Bsa*HI restriction enzymes) (Steingrube et al. 1995b). Rodrıguez-Nava et al. identified 44 strains of *Nocardia* by the same method (Rodríguez-Nava et al. 2006). Conville et al. identified 28 clinical isolates by RFLP analysis of *hsp65* and partial 16S rRNA, including *N. asteroides* type strain, *N. asteroides* (I), *Nocardia brasiliensis*, *N. nova* variant, *N. asteroides* (VI), *N. nova*, *Nocardia pseudobrasiliensis*, *N. transvalensis*, *N. farcinica*, *N. otitidiscaviarum,* and *N. asteroides* (II) (Conville et al. 2000). The cause of various RFLP patterns in the *hsp65* and 16S rRNA genes of *Nocardia* is DNA sequence heterogeneity, presumably leading to more than one RLFP pattern in a species (Brunello et al. 2001). Therefore, other molecular methods, such as full gene sequencing of the 16S rRNA gene, are necessary for conformation. *N. transvalensis* first was isolated from mycetoma in 1927. Until 1990, only a few strains of this species had been reported. In 1978, Gordon et al.


M20
M21
M15
M14
M13
M12
M11
M6
M5
M3
M2
M1
N. cyriacigeorgica Geno I DSM 44484; accession number: NR_041857
N. cyriacigeorgica Geno II DSM 43005; accession number: AF430020
M24
M26
M27
M28
M30
N. brasiliensis DSM 43758; accession number: AF430038
N. transvalensis DSM 43405; accession number: AF430047
M22
N. puris DSM 44599T; accession number: AJ508748
N. farcinica Geno I DSM 43665T; accession number: AF430033
N. farcinica Geno II DSM 44562; accession number: AF430034
M29
N. farcinica ATCC 3318T; accession number: Z36936
M10
N. nova Geno II DSM44561; accession number: AF430030
M25
N. nova Geno I JCM 6044T; accession number: AF430028
N. nova Geno III DSM44560; accession number: AF430029
N. nova Geno V DSM 40806; accession number: AF430032
N. nova Geno IV DSM 43256; accession number: AF430031
N. pseudobrasiliensis DSM 44290 T; accession number: AF430042
N. seriolae DSM 44129; accession number: AF430039
N. otitidiscaviarum Geno I DSM 43242 T; accession number: AF430067
N. otitidiscaviarum Geno II DSM44565; accession number: AF430068
M4
M16
M17
M18
M19
M23
N. asteroides ATCC19247T
0.002

◀ **Fig. 1** Full gene sequencing (1500-bp fragment) of the 16S rRNA gene based phylogenetic tree of *Nocardia* isolates with those of closely related species, computed by the Neighbor-joining (NJ) analyses and kimura 2-parameter (K2P) model. *Bar* 0.002 indicates one nucleotide substitution per 100 nucleotides

reported 5 isolates of this species using phenotypic methods (Gordon et al. 1978). In 1997, Wilson et al. identified 56 clinical isolates of *N. transvalensis* using phenotypic testing and antibiotic susceptibility testing (Brown-Elliott et al. 2006). The current study is the first report of *N. transvalensis* isolated from sputum in Iran. From 1992 to date, 8 cases of *N. transvalensis* have been reported from Asia (Kageyama et al. 2004; Wakamatsu et al. 2011; Ichinomiya et al. 2014; Nampoory and Khan 1997; Poonwan et al. 1995; Alp et al. 2006; Poonwan et al. 2005; Karakan et al. 2007). After pulmonary nocardiosis, skin and soft tissues are the most common sites of infection. Culture of the specimen, Gram stain, and modified acid-fast stain are important methods for the diagnosis of nocardiosis (Asilian et al. 2006). Cutaneous nocardiosis is still rare (Hironaga et al. 1990). Administration of corticosteroids and immunosuppressive drugs is a risk factor for opportunistic infections (Jaffe et al. 1999; Pallin

Table 5 Alignment of selected stretches of 16S rRNA gene of strains of some *Nocardia cyriacigeorgica* isolated from clinical samples with those of reference strains. All positions were defined based on *E. coli* (JO1695) numbering system

Bacterium	98	116	156	178	191	261	1001	1038	1251	1453	1454	1473	1476	1477	1480
N. cyriacigeorgica Geno I DSM 44484	A	A	T	G	C	T	C	G	G	T	G	T	G	G	A
N. cyriacigeorgica Geno II DSM 43005	A	A	T	G	G	T	C	G	G	C	G	T	A	G	A
M1	.	.	.	.	G	.	.	.	.	C	.	.	G	.	.
M2	G	.	.	C	G	G	G	C	.	C	.	.	–	.	.
M3	.	T	.	.	G	.	.	.	.	C	.	.	–	A	G
M5	.	.	G	.	G	.	.	.	A	C	–	ND	ND	ND	ND
M6	.	.	.	.	G	.	.	.	.	–	.	ND	ND	ND	ND
M11	.	T	.	.	G	.	.	.	.	C	T	G	A	.	.

(–) Means deletion, ND (not determined) and (.) means identical to those of 16S rRNA gene sequence of *N. cyriacigeorgica* Geno I, II

Table 6 Alignment of selected stretches of 16S rRNA gene of strains of *Nocardia otitidiscaviarum* isolated from clinical samples with those of reference strains. All positions were defined based on *E. coli* (JO1695) numbering system

Bacterium	114	173	189	547	850	922	1267	1413	1439	1455	1479	1495	1505
N. otitidiscaviarum Geno I	T	T	A	A	T	G	C	A	G	–	G	G	T
N. otitidiscaviarum Geno II	T	T	A	A	T	G	T	A	G	–	G	G	T
M4	.	.	.	.	.	.	C	.	.	G	.	.	.
M16	.	.	–	–	.	.	C	.	.	.	.	.	.
M17	.	.	.	.	.	.	T	.	.	G	–	A	A
M18	.	.	.	.	.	.	C	.	.	.	.	.	.
M19	–	–	–	–	.	.	C	.	.	.	.	.	.
M23	.	.	.	.	–	C	C	–	T	.	.	.	.

(–) Means deletion and (.) means identical to those of 16S rRNA gene sequence of *N. otitidiscaviarum* Geno I and II

Table 7 Alignment of selected stretches of 16S rRNA gene of strain of *Nocardia farcinica* isolated from clinical samples with those of reference strains. All positions were defined based on *E. coli* (JO1695) numbering system

Bacterium	95	619	895	1386	1498
N. farcinica ATCC 3318T	T	C	G	A	C
N. farcinica Geno I DSM 43665T	T	T	G	G	C
N. farcinica Geno II DSM 44562	C	T	G	G	C
M10	T	T	C	G	T
M29	ND	T	G	A	ND

(–) Means deletion, ND (not determined) and (.) means identical to those of 16S rRNA gene sequence of *N. farcinica*

et al. 2008) and is associated with nocardiosis; thus, the mortality rate in these patients is high (Kofteridis et al. 2005; Mishra and Randhawa 1969). Manifestations of cutaneous nocardiosis include abscesses, pustules, cellulitis, ulcers, and lymphocutaneous (Kofteridis et al. 2005; Hironaga et al. 1990). The diagnosis and clinical manifestation of cutaneous nocardiosis are laborious and nonspecific and it may be misdiagnosed as tuberculosis, actinomycosis, or streptococcal or staphylococcal infection (Kofteridis et al. 2005; Ambrosioni et al. 2010; Hironaga et al. 1990). Aerial hyphae formation is a differential diagnosis between *Nocardia* and *Mycobacterium* (McNeil and Brown 1994; Goodfellow 1973a). Primary cutaneous nocardiosis contains pus and granulomatous inflammation that is inoculated by soil and the environment (Hironaga et al. 1990; Shimizu et al. 1998). Cutaneous nocardiosis may be caused by *N. brasiliensis*, *N. asteroides*, *N. farcinica*, *N. transvalensis*, *N. otitidiscaviarum*, and *N. nova* (Shimizu et al. 1998; Kofteridis et al. 2005; Hironaga et al. 1990). In the literature, *N. brasiliensis* is the most common cause of skin infection (Saubolle and Sussland 2003). In a study by Kahn et al. (1981), a patient with diabetes mellitus reported *N. asteroides* primary cutaneous nocardiosis (Kahn et al. 1981). In 1990, Hironaga et al. reported *N. brasiliensis* in a girl's right knee (Hironaga et al. 1990). In 2003, Inamadar et al. reported *N. brasiliensis* and *N. nova* from primary cutaneous nocardiosis (Inamadar and Palit 2003). In another study by Kofteridis et al. (2005), *N. brasiliensis* was reported in 2 patients with polymyositis and chronic immune thrombocytopenic purpura with primary cutaneous nocardiosis who were treated with trimethoprim/sulfamethoxazole (Kofteridis et al. 2005). A study by Shimizu et al. in 2001 reported *N. nova* in the left hand of a healthy woman (Shimizu et al. 2001). In a study by Akasaka et al. (2011), *Nocardia araoensis* were isolated from systemic lupus erythematosus (Akasaka et al. 2011). A study by Saoji et al. reported *Nocardia* spp. in a healthy male in 2011(Saoji et al. 2012). In a study by Jaffe et al., *N. asteroides* was isolated from a breast abscess in a 60-year-old woman (Jaffe et al. 1999). Another study by Ichinomiy et al. isolated *N. transvalensis* from a mycetoma (Ichinomiya et al. 2014). Vanegas et al. isolated *N. brasiliensis* from femorotibial osteomyelitis and patients treated with discharge of infection and trimethoprim/sulfamethoxazole (Vanegas et al. 2014). *N. otitidiscaviarum* was first isolated from a guinea pig in 1924 and this organism was first reported in a human in 1974 (Sharma et al. 2007). *Nocardia otitidiscaviarum* is a Gram-positive and catalase positive organism with irregularly shaped branching (Mereghetti et al. 1997) that is resident in soil (Sharma et al. 2007). Mereghetti et al. reported *N. otitidiscaviarum* in 1997 in a skin wound (Mereghetti et al. 1997). A study by Sharma et al. in 2007 reported *N. otitidiscaviarum* in a patient with sickle cell anemia (Sharma et al. 2007). A study from China by Chen et al. in 2011 isolated *N. otitidiscaviarum* and *Pseudozyma aphidis* of a mycetoma (Chen et al. 2011). In a study from Italy, *N. otitidiscaviarum* was isolated from an AIDS patient in 1994 (Castelli et al. 1994). In another study by Candel et al. (2005), *N. otitidiscaviarum* was isolated from a bacteremia (Candel et al. 2005). In a study by Clark et al. (1995), *N. otitidiscaviarum*

Table 8 Alignment of selected stretches of 16S rRNA gene of strain of *Nocardia transvalensis* isolated from clinical samples with those of reference strains. All positions were defined based on *E. coli* (JO1695) numbering system

Bacterium	61	185	186	188	192	194	195	196	197	198	201	456	1005	1006	1007	1009	1020	1022	1025	1026	1036	1133	1137	1141
N. transvalensis DSM 43405T	–	–	–	C	T	G	C	A	T	G	G	A	A	A	G	T	A	C	C	C	T	G	A	C
N. transvalensis IFM 0998	G	A	C	–	–	A	T	G	–	–	T	T	C	G	C	G	C	G	T	T	C	A	G	T
M22	–	–	–	C	T	G	C	A	T	G	G	A	A	A	G	T	A	C	C	C	T	G	A	C

(–) Means deletion and (.) means identical to those of 16S rRNA gene sequence of *N. transvalensis*

was isolated from a primary cutaneous infection (Clark et al. 1995). Hashemi-Shahraki et al. reported *N.* otitidiscaviarum, *N. asteroides, N. nova*, and *Nocardia wallacei* from leg abscess, wound infection, soft tissue biopsy, and leg discharges, respectively in 2015 (Hashemi-Shahraki et al. 2015). Our study is the first report of isolates of *N. nova*, *N. otitidiscaviarum*, *N. farcinica,* and *N. cyriacigeorgica* from a wound, muscle abscess, thigh abscess (Behçet's disease), and breast abscess (Pemphigus disorder) from Iranian patients. The mortality rate in cerebral nocardiosis is 30 %, in contrast to 10 % for other abscess-causing bacteria (Tamarit et al. 2012). *Nocardia* infection imported into the CNS (central nervous system) is a primary infection and the mortality rate is high (90 %) in patients with cerebral abscess (Zakaria et al. 2008). In 2003 in a study by Yorke et al. *N. transvalensis* was isolated from a brain abscess (Yorke and Rouah 2003). A study by Liu et al. in 2004 from Taiwan isolated *N. abscessus* from brain abscess (Liu et al. 2011a). In a study by Barnaud et al. (2005), a patient with HIV reported *N. cyriacigeorgica* (Barnaud et al. 2005). In another study, two cases of *N. farcinica* from brain abscess were reported in Japan (Izawa et al. 2011). El Hymer et al. reported *N. asteroides* in 2011 in an immunosuppressed patient with a brain abscess (El Hymer et al. 2011). Another study by Tamarit et al. in 2012 reported *N. asteroides*, *N. farcinica*, *Nocardia arthritidis* and *Nocardia cerradoensis* in 4 patients with brain abscesses (Tamarit et al. 2012). Eshraghi et al. (2014) isolated *N. cyriacigeorgica* of brain abscess from Iran (Eshraghi et al. 2014). A study by Hashemi-Shahraki et al. (2015) reported *N. cyriacigeorgica* and *Nocardia carnea* from brain abscess in Iran Hashemi-Shahraki et al. 2015). The present study is the first report of *N. farcinica* and *N. otitidiscaviarum* in two patients with brain abscess from Iran.

Conclusions

In summary, reports of *Nocardia* infections are rare and *Nocardia* species are causing various illnesses that manifest with disparate clinical signs; therefore, the identification of *Nocardia* spp. using various phenotypic tests and molecular methods is important for epidemiology and treatment.

Table 9 Alignment of selected stretches of 16S rRNA gene of strain of *N. nova* isolated from clinical samples with those of reference strains. All positions were defined based on *E. coli* (JO1695) numbering system

Bacterium	140	187	201	381	457
N. nova Geno I JCM 6044T	C	G	T	A	A
N. nova Geno II DSM44561	T	G	T	A	A
N. nova Geno III DSM44560	C	G	T	G	A
N. nova Geno IV DSM 43256	C	G	T	A	G
N. nova Geno V DSM 40806	C	T	G	A	A
M25	C	G	T	A	A

(–) Means deletion and (.) mean identical to those of 16S rRNA gene sequence of *N. nova*

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