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Report of *Nocardia* species isolated from soil by *16S rRNA* gene sequencing method in Isfahan, Iran

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Abstract

Introduction: The genus *Nocardia* belongs to aerobic *Actinomycetes*. They are a large group of soil-dwelling bacteria that are distributed worldwide. Using various key conventional and molecular diagnostic tests, several *Nocardia* isolates were identified, characterized and distinguished.

Materials and methods: In our study, soil isolation method was Slip-buried type and DNA extraction was obtained through Microwave oven method and *Nocardia asteroides* DSM 43757-type strain as standard was tested to approve the above method. Following this study, Polymerase Chain Reaction was carried out using universal primers.

Results: Phenotypic and molecular data analysis, particularly *16S rRNA gene* sequencing, provided evidence on *Nocardia cyriacigeorgica* KC577151, *Nocardia asteroides* KC577152, *Nocardia cummidelens* KC577153, *Nocardia asteroides* KC577154, *Nocardia asteroides* KC577155, *Nocardia coubleae* KC577156 involvement in Iran soil in Isfahan and these isolates were eventually registered in NCBI gene bank.

Discussion and conclusion: In this research, six *Nocardia* species were isolated and identified some of which isolated species were novel in Iran soil in Isfahan, at the time of isolation and detection. To identify more species of *Nocardia* in subsequent studies, proliferation and sequencing of other genes of the bacteria such as *hsp65*, *ITS*, *secA*, *rpoB* and *sod* can be applied.

Key words: Novel *Nocardia* species, *16S rRNA* sequencing, Soil samples, Isfahan, Iran

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Introduction

The genus *Nocardia* belongs to the family Nocardiaceae, and members of the genus are all aerobic, Gram-positive, modified acid fast and non-motile actinomycete that form filamentous branched cells which fragment into pleomorphic rod-shaped or coccoid elements, non-spore-forming (1 & 2) catalase and urease positive bacteria that belong to *Actinomycetes* group (3). Soil is one of the best sources for maintaining *Nocardia*. However, these organisms are found in environments such as air, water, plants and rotten materials. *Nocardia* may be acquired in dry, dusty and often windy locations (2). *Nocardia* species are associated with opportunistic infections in human and animals that might become fatal. Anyway, some species can infect both immune-compromised and immune-competent individuals (4).

The genus is comprised of 105 acknowledged species according to the National Center for Biotechnology Information (NCBI) and German Collection of Microorganisms and Cell Cultures is called DSMZ. The process of using phenotypic and biochemical methods to identify *Nocardia* is difficult and tedious.

Molecular techniques were developed in the 1990s, including a *16S rRNA* gene PCR-based. Molecular techniques were developed in the 1990s, including a *16S rRNA* gene PCR-based technique for distinguishing the genus *Nocardia* among aerobic actinomycetes (5 & 6).

Members of these taxa organize a different phyletic line in the *16S rRNA* tree that can be distinguished from one another by using a combination of morphological and biochemical features (7). *rRNA* genes are used to approximate evolutionary history and taxonomic designation of individual organisms in a wide range. The choice of *rRNA* genes as optimal tools for such purposes are based on both observations and presumptions of ribosomal conservation. *rRNA* genes are necessary components of the ribosome, which include 50 proteins and three classes of RNA molecules. In bacteria, three *rRNA* genes are categorized into a gene cluster which is expressed as a single operon and may be present in multiple copies in the genome (8). The *16S rRNA* is nearly 1, 500-nucleotides sequence encoded by the *16S ribosomal RNA* (*rRNA*). The sequence is an extremely conserved gene in which common areas covered in all living beings existed while nucleotide variations are concentrated on specific regions and also the hyper variable regions, which are defined by variability of specific species (9). To recognize various species of microorganisms that use *16S rRNA* hyper-variable regions called V1- V9, sequence analysis of the *16S rRNA* gene was used to identify bacterial species and achieve taxonomic studies in a wide range (10 & 11).

Unfortunately, *16S rRNA* hyper-variable regions perform different degrees of sequence diversity, and hyper-variable regions cannot distinguish all bacteria solitarily, and molecular diagnostic techniques, such as real-time PCR or melting temperature analysis, must be used (11). This paper was based on identification of *Nocardia* species by *16S rRNA* gene sequencing, some of which were first

reports from Iran soil at the time of isolation and detection, Such as *N. coubleae, N. cummidelens and N. cyriacigeorgica*. Then data regarding these isolates were entered in NCBI Database by Sequin software, and phylogenic tree and similarity matrix were drawn for isolates.

Materials and methods

A total of 70 soil samples were collected from several different locations in Isfahan's suburb hospital areas, parks, agricultural lands, gardens and arid lands at different months of year. Fifty-gram of soil samples were collected from 3 cm to 5 cm depth. Soil samples temperature and the pH were measured. All samples were stored at low temperature (4°C) until they were tested (2).

Isolation methods: Slip-buried Method and Some characteristics of Phenotypic include the following: 3-5 g of soil was added to 10 ml normal saline. Tubes were shaken for 3 min and the suspensions were incubated for 15 min in room temperature. 3-5 ml of the supernatant solution was transferred to another sterile tube by sterile pipette.

The streptomycin/ chloramphenicol solution (half of the total volume) was added to the supernatant. The mixture was incubated for ½ h. One drop (0.05 ml) of each sample was cultured on BHI agar with 5% human blood medium (12 & 13) and checked for hemolysis. Cycloheximide (0.5 g/l) and kanamycin (25 mg/l) was added to the tube or plate immediately. They were shaken and were incubated at 37°C for 2 weeks (2), Afterwards some of the Phenotypic characteristics were studied (7 & 14) such as: Modified acid fast, Growth in Lysosyme, Hydrolysis of Tyrosine, Xanthine, Hypoxanthine and Uric acid.

DNA extraction techniques: In this study, DNA extraction method was Microwave oven method (15) and as mentioned before, it was tested with *Nocardia asteroides* DSM 43757-type strain as standard. In Microwave method, based on studies done by Salgado *et al*., the suspension was made with a single bacterial colony in 20 µl of deionized water subjected to 800 watt (W) microwave oven for 10, 15 and 20 seconds (S) at different potencies: 80, 160, 240, 320, 400, 560, 720 and 800 watts. An electrophoresis with agarose Gel 1% was applied to survey the presence and quality of the extracted DNA. In the present study, a suspension was made with single bacterial colony in 70 µl of deionized water. Then, suspensions were treated in a microwave oven for 10, 13, 20 seconds at 360 and 540 watts. Subsequently, the suspensions were put in -20°C.

Polymerase Chain Reaction (PCR) technique for *16S rRNA* gene: PCR was implemented according to the previously explained (16 & 17), using universal Forward primer: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3' and Reverse primer: 1492R:5'-GGTTACCTTGTTACGACTT-3'. These 1500 bp PCR products were amplified by 27F and 1492R primers. PCR was carried out by using Prime Taq Premix (2X) kit (GENET BIO). In a final volume of 20 µl (10 µl 2XPrime Taq Premix, 1 µl F primer, 1 µl R primer, 6 µl sterilized deionized water), 2 µl of DNA was extracted. Amplification was done in thermal cycler

(Eppendorf) programmed as the following: 5 min initial denaturation step at 94^0C, pursued by 30 cycles (94^0C for 30 Sec, 58^0C for 30 Sec, 72^0C for 60 Sec), and a 5 min final extension step at 72^0C. The bands were evaluated in position of 1500 bp by 1% agarose gel electrophoresis. In the last step, *16S rRNA* gene sequencing (11 & 18) and its analysis was done with proper software (11).

Molecular detection and identification of *Nocardia* isolates using sequencing of *16S rRNA*: For precise identification of *Nocardia* isolates, all of microorganisms were identified using PCR of a 1500-bp region of the *16S rRNA* gene. Then, the samples were sequenced. *16S rRNA* gene standard sequences of various species of *Nocardia* were received from international databases (NCBI) and the sequences of the isolates obtained in this study were aligned in the CLC Main Workbench 6 software. BLASTN was done on Forward (F) sequences and Reverse complement (RR) sequences in the database of NCBI. After the BLAST, the Forward Reverse complement sequences and their related dendrogram, which the company had sent, were compared with each other.

Positioning was regarded manually through positions of standard sequence (subject) close to the species obtained in this study which were obtained from NCBI databases. Forward sequences and Reverse complement sequences were assembled manually in the Microsoft Word. After assembling these sequences in the NCBI, BLASTN were blasted and in a moment, they could be examined in terms of percentage of similarity with the bacteria species close to identified species in this study. Bacteria species, which had greater Max identity, Query coverage, less Gap and sequences with E-values close to zero are as our considered sequences in this research. The corrected sequences were entered and then aligned in the CLC Main Workbench 6 software. Next, Fasta format were made for entery of the genes in the NCBI Gen Bank, phylogenetic tree and Matrix similarity. Nucleotide sequences accession numbers: *N. cyriacigeorgica*, *N. asteroides*, *N. cummidelens*, *N. asteroides*, *N. asteroides*, *N. coubleae* sequences in this study were submitted to the GenBank under the accession numbers KC577151, KC577152, KC577153, KC577154, KC577155, KC577156 for the *16S rRNA* gene respectively.

Results

The initial isolation was identified by phenotypic isolation methods, such as Modified acid fast, Growth in lysozyme, Hydrolysis of Tyrosine, Xanthine, Hypoxanthine and Uric acid (19).

To identify more precisely, PCR-Sequencing was performed for isolates of *16S rRNA* gene. A total of six isolates were obtained via the *16S rRNA* gene sequencing. *16S rRNA* gene sequences recorded in GenBank of NCBI and Accession numbers were received. Nucleotide sequences accession numbers as mentioned are in the following. *N. cyriacigeorgica*, *N. asteroides*, *N. cummidelens*, *N. asteroides*, *N. asteroids* and *N. coubleae* sequences in this study were submitted to the GenBank under the accession numbers KC577151, KC577152, KC577153, KC577154, KC577155, and KC577156 for the *16S rRNA* gene

respectively. In the following, isolated strains, *N. cyriacigeorgica* KC577151, *N. asteroides* KC577152, *N. cummidelens* KC577153, *N. asteroide* KC577154, *N. asteroides* KC577155, and *N. coubleae* KC577156 were compared with NCBI closely related type species, such as *N. cyriacigeorgica* DSM 44484T, *N. asteroides* DSM 43757T, *N. cummidelens* DSM 44490T, *N. asteroids* DSM 43757T, *N. asteroides* DSM 43757T, and *N. coubleae* OFN N11 (data not shown), and these results were found 100, 100, 97, 100, 100 and 99% similarity with these strains and gaps in all of the isolated strains were 0%, respectively. The phylogenetic tree was constructed with the MEGA5 software package with tree algorithms (Fig. 1), namely the neighbour-joining (20). The tree topology was determined by using 1000 bootstrap datasets. The evolutionary distances were computed using the Jukes-Cantor method (21).

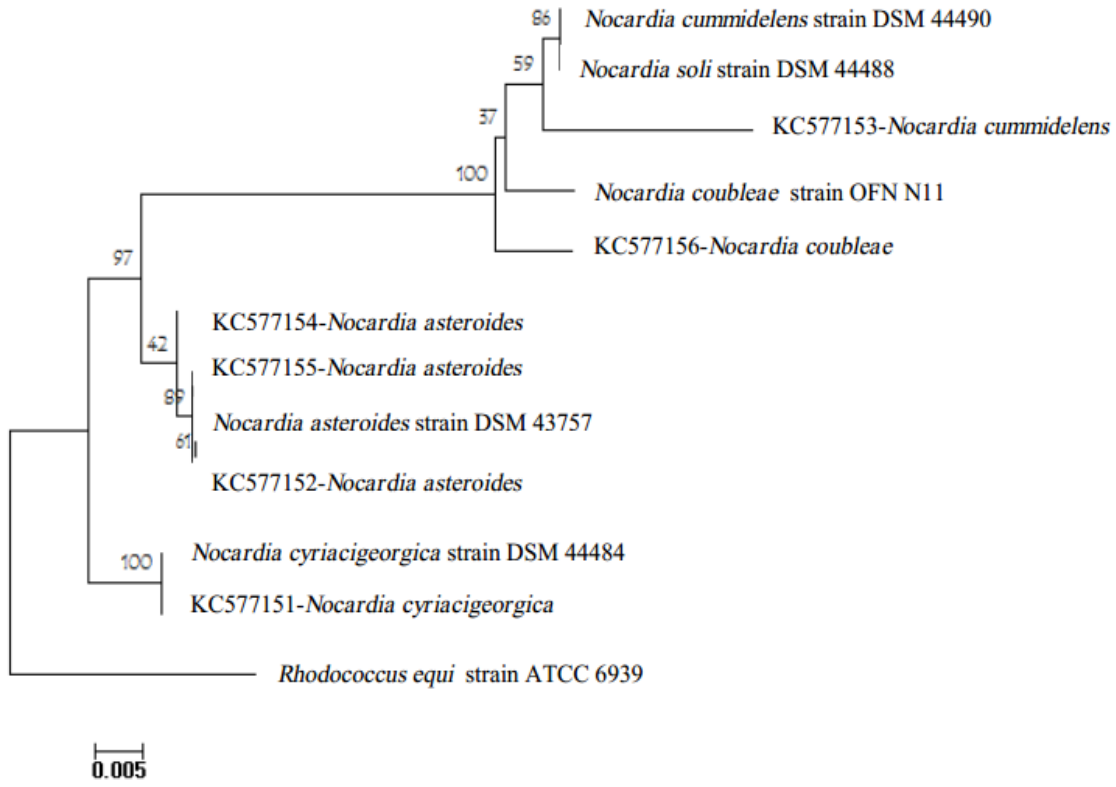

Fig. 1: Neighbor-joining tree, inferred using MEGA 5 (20 & 22), based on *16S rRNA* gene sequences available from GenBank NCBI (accession numbers are shown in tree), as determined from 1000 bootstrap samples (1000 replicates), is indicated by percentages at each node The scale bar indicates 0.005 substitution per nucleotide position. The evolutionary distances were computed using the Jukes-Cantor method (21) and are in the units of the number of base substitutions per site. There were a total of 1501 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (23). Afterwards, Similarity values for *16S rRNA* gene sequences of isolates of *Nocardia* with that of closely related type species was depicted by CLASTAL2.1 Multiple Sequence Alignments Server[1].

Table 1: Similarity values for *16S rRNA* sequences of isolates of *Nocardia* with that of closely related type species by CLASTAL2.1 Multiple Sequence Alignments Server.

	% Similarity to										
Taxon	1	2	3	4	5	6	7	8	9	10	11
1. *N. asteroides* DSM 43757	-										
2. *N. cyriacigeorgica* DSM 44484	98.53										
3. *N. cummidelens* DSM 44490	97.45	96.72									
4. *N. soli* DSM 44488	97.45	96.72	100.00								
5. *N. coubleae* OFN N11	96.75	96.14	98.41	98.41							
6. *N. cyriacigeorgica* (KC577151)	98.27	100.00	96.54	96.54	95.08						
7. *N. cummidelens* (KC577153)	93.44	91.90	96.51	96.51	96.23	91.90					
8. *N. asteroides* (KC577155)	100.00	98.39	97.29	97.29	95.84	98.24	93.44				
9. *N. coubleae* (KC577156)	95.22	94.56	97.89	97.89	98.56	94.56	82.26	95.22			
10. *N. asteroides* (KC577152)	100.00	98.36	97.24	97.24	95.69	98.36	93.44	100.00	95.22		
11. *N. asteroides* (KC577154)	100.00	98.32	97.17	97.17	92.36	98.32	89.80	100.00	95.22	98.24	-

Discussion and conclusion

The aim of this study was isolation, phenotypic and molecular identification of various *Nocardia* species from soil samples of Isfahan suburb hospitals. Since these organisms are widely distributed in environment and fragmented nocardial cells may be scattered and increased their acquisition through the respiratory path and favor traumatic inoculation in both immune-compromised and immune-competent hosts, and as they may sometimes be mistaken with tuberculosis, even these bacteria may be fatal. Thus, we decided to isolate these bacteria from surrounding soils of Isfahan hospitals to find a connection between environmental isolates from clinical isolates of these bacteria in future, and then keep patients under more intensive care (2 & 24). At the time of the study, other researches which had been done with regard to this bacteria in Iran were mostly on clinical isolates or those merely isolated by phenotypic tests, which are mentioned in the following. In the present study, as mentioned, Slip-burid-method was surveyed for isolation of *Nocardia* from soil. Indeed, in our study *N. cyriacigeorgica* KC577151, *N. asteroides* KC577152, *N. cummidelens* KC577153, *N. asteroide* KC577154, *N. asteroides* KC577155, *N. coubleae* KC577156 species were isolated from Isfahan, Iran soil samples. Herein, at the time of the study, *N. cyriacigeorgica* KC577151, *N. cummidelens* KC577153, and *N. coubleae* KC577156 were reported for the first time from Isfahan, Iran soil. Due to high similarity in *16S rRNA* gene variable regions, *Nocardia cummidelens*

were not discriminated from *Nocardia soli*. The following several studies refer to this field. In a study done by Trivisan et al. in 1889, *Nocardia asteroides* were isolated from clinical specimens. In 2001, a study done by Corring Yassin presented *Nocardia ciriacigeorgica* from bronchial secretions in Germany (25) as well as in 2011, Shojaei reported this species from clinical specimens in Iran. In Shojaei, full sequence of *16S rDNA* and partial sequence of *hsp65* were used. *16S rDNA* showed 100% similarity with *N. cyriacigeorgica* DSM 44484T and *N. asteroides* typeVI and *hsp65* gene sequence showed 100% similarity with *N. cyriacigeorgica* DSM 44484T and 99.55% with *N. asteroides* typeVI. (26). In 2001, Maldonado spotted *Nocardia cummidelens* from soil and water in England. In Maldona findings, According to chemical and morphological features, Twenty-eight isolates were *nocardiae*. These organisms formed a monophyletic clade in the *16S rDNA* tree with *Nocardia salmonicida*. Due to genotype data, three of the strains, isolates S1, W30 and R89 were distinguished from one another. These organisms were called *Nocardia cummidelens* sp. nov., *Nocardia fluminea* sp. nov. And *Nocardia soli* sp. nov. respectively (27). In 2007, Rodriguez Nava reported *Nocardia coubleae* from oil-contaminated soil in Kuwait, in which they detected two bacterial isolates (OFN N11 and OFN N12^T) based on a multi-genic method that included *16S rRNA*, hsp65 and sod gene sequencing. The closest species was *Nocardia ignorata* (with 99.4, 99.5 and 98.6% gene sequence likeness to *16S rRNA*, *hsp65* and *sod* genes, respectively). In their study, novel isolates were recognized phenotypically from the type strains of the genus *Nocardia*. The novel isolates had 26% relatedness to type strain of *N. ignorata* in DNA–DNA hybridization experiments. On the basis of genotypic and phenotypic methods, two novel species were named *Nocardia coubleae* sp. nov. with type strain OFN N12T (14). In a 2012 study by Kachuei, *Nocardia was* presented *in Isfahan, Iran*. They studied on *Nocardia* with conventional biochemical tests and physiological Characteristics (14). In Kachuei's study, from 153 (19.1%) *Nocardia* isolates identified, *Nocardia asteroids* complex (45.5%) and *Nocardia brasiliensis* (24.7%) were the most frequently isolated species, followed by *Nocardia otitidiscaviarum* (2.2%), *Nocardiopsis dassonvillei*, *Actinomadura actinomadura* (each 1.7%) and *Nocardia transvalensis* (1.1%) and also unknown spp. (23.0%) (2). In the present study, *Nocardia cyriacigeorgica, Nocardia asteroides, Nocardia cummidelens*, *Nocardia coubleae* were isolated from Isfahan soil. Among these species, *Nocardia cyriacigeorgica, Nocardia cummidelens*, and *Nocardia coubleae* were isolated from Isfahan soil, Iran for the first time and their *16s rRNA* genes were submitted to the Gene Bank of NCBI database and were recorded there. Afterwards, relevant phylogeny or evolutionary tree was depicted using Molecular Evolutionary Genetics Analysis

version 5 (MEGA5) Software (20) based on *16S rRNA* gene sequences available from NCBI Gene Bank. K Tamura (23) and Patrycja Golinska (28) also did analysis using MEGA5 Software. Then, similarity values were plotted for *16S rRNA* sequences of isolates of *Nocardia* with that of closely related type species by CLASTAL2.1 Multiple Sequence Alignments Server. While *Nocardia* species genetic diversity was demonstrated using the NJ phylogenetic tree generated from the *16srRNA* sequences, as determined from 1000 bootstrap samples (1000 replicates), and indicated by percentages at each node, the scale bar indicates 0.005 substitution per nucleotide position. To summarize in this research, we isolated three *Nocardia* novel species for the first time from Isfahan soil by PCR-Sequencing. Moreover, their *16s rRNA* genes were submitted to the Gene Bank of NCBI database and were recorded there.

Consequently, we suggest for better identification of different species of this bacteria, Appling the sequencing *16S rRNA*, *hsp65*, *ITS*, *secA*, *rpoB* and *sod* genes; moreover, analyzing the bacteria cell wall by using relevant technique, such as HPLC, but it cost you a lot of money.

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[1]- https://www.ebi.ac.uk/tools/msa/clustalw2/ (Table 1).

گزارش گونه‌های نوکاردیای جداسازی شده ازخاک، توسط تعیین توالی ژن *16S rRNA*، اصفهان، ایران

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چکیده

مقدمه: جنس نوکاردیا جزو اکتینومیست‌های هوازی هستند که یک گروه بزرگ از باکتری‌های ساکن خاک را شامل می‌شوند، که در جهان پخش شده‌اند. در این مطالعه، با استفاده از آزمون‌های تشخیصی رایج، مرسوم و مولکولی گوناگون، چندین ایزوله‌های نوکاردیا جداسازی و تشخیص داده شد.

مواد و روش‌ها: در این پژوهش، برای جداسازی از خاک، روش Slip-buried و برای استخراج DNA، روش Microwave oven استفاده شد و در این روش‌ها، سویه استاندارد *Nocardia asteroides* DSM 43757 استفاده شد. در ادامه، روش PCR با استفاده از پرایمرهای عمومی انجام شد.

نتایج: با تحلیل اطلاعات فنوتیپی و مولکولی، به‌ویژه تعیین توالی ژن *16S rRNA*، ژن‌های نوکاردیای‌های جداسازی شده از خاک اصفهان، که در ادامه به آن‌ها اشاره شده است، درپایگاه داده NCBI باAccession number مربوط به هر ژن ثبت شدند. نوکاردیاهای ثبت شده در NCBI:

Nocardia cyriacigeorgica KC577151, *Nocardia asteroides* KC577152, *Nocardia cummidelens* KC577153, *Nocardia asteroides* KC577155, *Nocardia asteroides* KC577155, *Nocardia coubleae* KC577156 . .

بحث و نتیجه‌گیری: در این پژوهش ۶ گونه نوکاردیا جداسازی و تشخیص داده شد، که برخی از این گونه‌های جداسازی شده از خاک ایران، اصفهان، در زمان جداسازی و تشخیص،گونه‌های جدیدی بودند. برای تشخیص بیشترگونه‌های مختلف نوکاردیا می‌توان در مطالعات بعدی از تکثیر و تعیین توالی ژن‌های دیگر این باکتری از جمله *ITS,hsp65 secA, rpoB* و *sod* بهره گرفت.

واژه‌های کلیدی: گونه‌های جدید نوکاردیا، توالی‌یابی *16SrRNA*، نمونه‌های خاک، اصفهان، ایران

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* نویسنده مسؤول مکاتبات
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