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# Diversity of *Nocardia* species isolated from transplantation and cancer centers of Tehran hospitals

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ABSTRACT

**Objective:** To identify the biodiversity of *Nocardia* species isolated from the environments of Tehran hospitals *via* phenotypic and PCR molecular methods.

**Methods:** A total of 236 samples were collected from the transplantation and cancer centers of five Tehran hospitals, and the presence of *Nocardia* was identified by using standard protocols. Conventional tests were used for preliminary identification, and PCR amplification of the 598 bp amplicon of the 16S rRNA and sequence analysis of 16S rRNA were performed for genus and species identification.

**Results:** From the 236 soil samples, 16 samples were identified as positive based on the culture tests, while 8 samples showed positive results based on the molecular method. The phylogenetic tree showed that the most frequent genus of *Nocardia* was related to *Nocardia cyriacigeorgica*.

**Conclusion:** The hospital environment is a potential reservoir of a diverse range of *Nocardia* species. Due to the strong capability of survival, these bacteria bring a threat to the health of patients. Its identification can help physicians to implement treatments and is important for healthcare centers.

## 1. Introduction

*Nocardia* is distinguished as a genus of bacilliform Gram-positive bacteria belonging to Actinobacteria, which can be found as an environmental saprophyte in soil, water, air, as well as decaying plants and animals around the world[1,2]. This partially acid-fast bacterium is also catalase-positive, non-motile, and non-capsulated[3,4]. Thus far, a total number of 75 species of *Nocardia* have been identified using phenotypic and molecular methods. Among them, 25 species have been classified as potential pathogens including *Nocardia asteroides* (*N. asteroids*) complex as the most common one[5]. Nocardiosis is normally manifested as acute or chronic symptoms along with infection and granulomatosis which can lead to invasive diseases in healthy and immune-suppressed individuals. Respiratory symptoms occurred in about 70% cases of nocardiosis which are mostly (80%) related to *N. asteroides* complex. Skin forms are also normally associated with *Nocardia brasiliensis* (*N. brasiliensis*)[6]. The prevalence of nocardial infection in individuals with acquired immune deficiency syndrome (AIDS) and immune-suppression including those taking corticosteroid drugs and transplant recipients is 100% compared with healthy individuals. Moreover, the duration of treatment is long and mortality rates are higher than 85%[7-9]. Besides, *Nocardia* is known as a component

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of the microflora of soil and it can be also found in dust and air all over the world. Some pathologic factors can be considered as predisposing to nocardiosis, including lymphoproliferative syndrome, neoplasia, AIDS, liver cirrhosis, organ transplants, and immunosuppressant drugs[10]. Intravenous drug abuse also seems to be one risk factor. Determining the biodiversity of opportunistic saprophytes in hospital settings can help diagnose and treat more quickly. Phonotypic methods containing macroscopic examination of clones, microscopic assessment of samples (using Gram staining, partially acid-fast, and observation of bacilliform bacteria), and culturing in selected environments are considered as references for *Nocardia* diagnosis. However, they are time-consuming and need suitable bacterial isolates. Molecular methods such as polymerase chain reaction (PCR) have been extensively used in diagnoses in recent years with higher specificity and sensitivity compared with phenotypic and microscopic methods and can make it possible to examine all known species of *Nocardia*[1,8]. There is no evidence of transmission of *Nocardia* from one person to another, but the presence of this bacterium in an environment, especially in soil and dust, along with its sustained survival in these settings allows it to easily invade their hosts. Transplantation and cancer centers of hospitals are favorite sites wherein these bacteria can invade patients. Due to immunodeficiency in these individuals, the presence of *Nocardia* can cause serious problems. The purpose of this study was to isolate and investigate the diversity of the species of this bacterium in the surrounding environments and the soil of the gardens of transplantation and cancer centers of hospitals located in the city of Tehran, Iran.

## 2. Materials and methods

### 2.1. Sampling

A total number of 236 soil samples were collected from the green spaces and gardens surrounding the buildings of transplantation and cancer centers of five hospitals in the city of Tehran. To conduct the sampling, 3 to 5 centimeters of dry soil from the green spaces and gardens were removed and enough soil amounts were sampled. Species of *Nocardia* from soil samples were isolated by the paraffin baiting technique. In this way, 10 g of soil sample was added to 15 mL carbon-free broth ($NaNO_3$ 2g, $ZnSO_4$ 2 mg, $MgSO_4 \cdot 7H_2O$ 0.5 g, $K_2HPO_4$ 0.8 g, $FeCl_3$ 10 mg, and $MnCl_2 \cdot 4H_2O$ 8 mg); then, the tubes were incubated at 37℃ for 21 d. Gypsum form colonies were accordingly cultured on brain heart infusion agar[11].

### 2.2. Conventional bacterial identification

The environmental isolates were identified primarily as *Nocardia* by conventional phenotypic tests including partial acid-fast staining, growth at 45℃, pigment production and standard biochemical assays, including resistance to lysozyme, hydrolysis of tyrosine, xanthine and hypoxanthine tests[12].

### 2.3. Molecular identification

The genome of the bacterium was extracted by a combination of boiling and alkaline hydrolysis. Then, the supernatant obtained from the extraction was analyzed quantitatively and qualitatively. Precipitated DNA was resuspended in 50 μL TE buffer and stored at 20 ℃. The genus was identified by PCR with primers NG1 and NG2. PCR was carried out by using Taq DNA Polymerase Master Mix RED (2×) kit (Ampliqon). In a final volume of 25 μL (12.5 μL Master Mix, 1 μL F primer, 1 μL R primer, 8 μL sterilized deionized water, 2.5 μL of DNA was extracted. Amplification was done in thermal cycler programmed as the following: 4 min initial denaturation step at 95℃ for 30 cycles (94℃ for 40 s, 52℃ for 45 s, 72℃ for 60 s), and a 5 min final extension step at 72℃. The bands were evaluated in position of 598 bp by 1% agarose gel electrophoresis (Table 1). The 16S rRNA PCR was identified by PCR with primers 27F and 1492R. Amplification was done in thermal cycler programmed as the following: 4 min initial denaturation step at 95℃ for 30 cycles (94℃ for 45 s, 54℃ for 45 s, 72℃ for 60 s), and a 10 min final extension step at 72℃. The bands were evaluated in position of 1 480 bp by 1% agarose gel electrophoresis. Finally, 16S rRNA sequencing using universal primer and PCR was utilized for the final identification and diagnosis of the species. Sequencing was performed by the Macrogen Company (South Korea), and the sequence data received were aligned manually with existing sequences of *Nocardia* retrieved from the GenBank database and analyzed using the Blast program in GenBank and the MEGA 7 program. The *N. brasiliensis* ATCC 700358 strain was also used as a positive control[13].

## 3. Results

### 3.1. Phenotypic diagnosis

Out of the 236 soil samples, 16 isolates were suspected to be related to *Nocardia* after an initial culture (Table 2). Of these, only 5 species were diagnosed with biochemical tests. In macroscopic

**Table 1.** Primers used in this study.

| Primers | Primer names | Sequence | Annealing temperature (℃) | Product length |
|---|---|---|---|---|
| Genus primers | NG1 | 5'-GGTTGTAAACCTCTTTCGA-3' | 52.51 | 598 bp |
| | NG2 | 5'-ACCGACCACAAGGGGG-3' | 57.89 | |
| 16S rRNA primers | 27F | 5'-AGAGTTTGATCCTGGCTCAG-3' | 56.92 | 1 480 bp |
| | 1492R | 5'-GGTTACCTTGTTACGACTT-3' | 52.20 | |

examination, colony color and appearance were observed. Through evaluation of growth ability in broth lysozyme, growth at 45℃ for 3 d, and biochemical tests, *N. asteroides*, *Nocardia cyriacigeorgica* (*N. cyriacigeorgica*), *Nocardia otitidiscaviarum*, *Nocardia cummidelens*, and *Nocardia ignorata* were identified (Table 3).

### 3.2. Molecular diagnosis and phylogenetic analysis of isolates

In this study, a total of 16 isolates were recovered from 236 soil samples collected from transplantation and cancer centers of Tehran hospitals 8 isolates were identified as *Nocardia* by Molecular method (Figure 1A and B). The phylogenetic tree is shown in Figures 2. The most frequent genus of *Nocardia* was related to *N. cyriacigeorgica*. The resulting sequence of the isolates had the highest phylogenetic similarity with type strains.

**Table 2.** Positive culture isolates from Tehran hospital soil sampels.

| Hospital name | Soil samples (*n*=236) | Positive culture (*n*=16) |
|---|---|---|
| Shariati | 58 | 4 |
| Imam khomeini | 58 | 6 |
| Taleghani | 40 | 3 |
| Valiasr | 40 | 1 |
| Mofid | 40 | 2 |

## 4. Discussion

Infection caused by *Nocardia* species received more attention in recent years. Over 17 novel species were isolated and identified from clinical samples in the last decade[14]. It should be noted that *Nocardia* is from the family of nocardiosis, which has been identified in 111 species over the past decades. Among them *N. asteroides*, *Nocardia farcinica*, *Nocardia cyriacigeorgica*, *Nocardia nova*, *N. brasiliensis*, *Nocardia otitidiscariarum*, *Nocardia pseudobasiliensis*, and *Nocardia transvalensis* have been usually reported in human infections. In recent years, new and varied types of human infections have been isolated, dominated by 17 new species, indicating the importance of this bacterium in infectious diseases. One of the most important features of *Nocardia* is its ability to survive in different environmental conditions, so it increases the risk of infection in patients. In areas surrounding hospitals, due to the presence of green spaces and gardens, nocardial agents in soil may enter into hospitals through dust and wind, which are considered as risk factors. It is lack of routine methods to identify these factors in hospitals. In the present study, a total of 16 clones were suspected to be related to *Nocardia*, and 8 cases of *Nocardia* were diagnosed as opportunistic pathogens of the nocardiosis family *via* phenotypic and molecular methods. Studies from Kazakhstan and the UK reported that the isolation

**Table 3.** Profile phenotypic features of *Nocardia* isolates from hospital environments.

| Tests | *Nocardia ignorata* | *Nocardia cummidelens* | *Nocardia cyriacigeorgica* | *Nocardia otitidiscaviarum* | *Nocardia asteroides* |
|---|---|---|---|---|---|
| Citrate | - | - | - | - | - |
| Nitrate reduction | + | + | + | + | - |
| Urease | + | + | - | + | + |
| Casein hydrolysate | - | - | - | - | - |
| Lysozyme resistance | + | + | + | + | + |
| Utilization of sorbitol | - | - | - | - | - |
| Hydrolysis of tyrosine | - | - | - | - | - |
| Hydrolysis of xanthine | - | - | - | + | - |
| Hydrolysis of hypoxanthine | - | - | - | + | - |
| Growth at 45 ℃ | - | - | + | Variable | - |
| Hydrolysis of esculin | + | - | - | + | - |

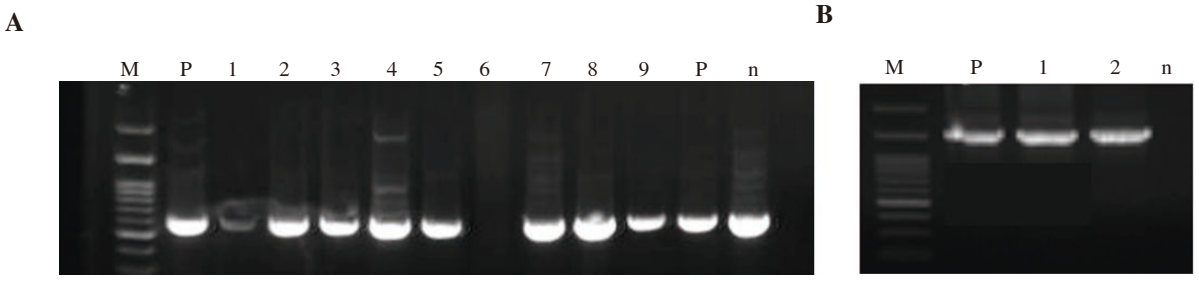


**Figure 1.** Electrophoresis of PCR products using NG1 and NG2 primers (A) and 16S rRNA PCR products using 27F, 1492R primers (B). M=100 bp marker, Lane P= positive control, Lane 0-9=isolates, *n*=negative control.

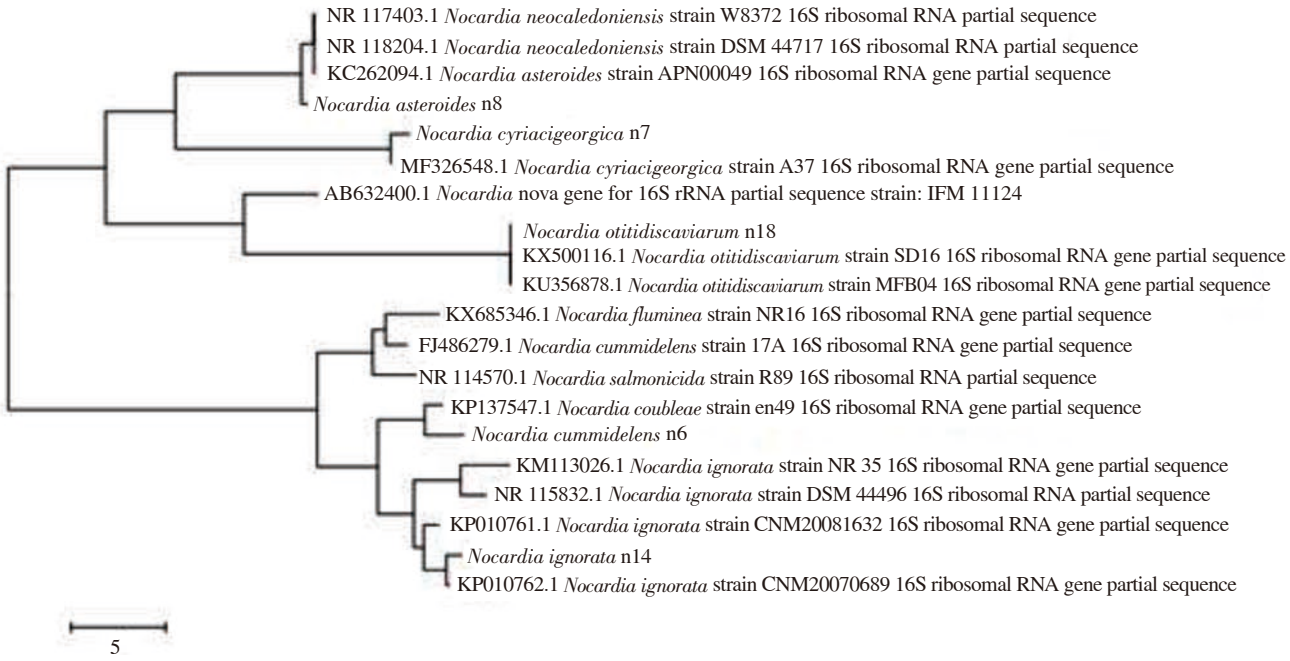


**Figure 2.** 16S rRNA sequence-based phylogenetic tree using the neighbor-joining method.

rate was 27.7%. The incidence was higher in Thailand but lower in Kuwait and Brazil[13,15,16]. The most frequent species found in this study was *N. cyriacigeorgica*, as an opportunistic bacterium isolated from chronic pulmonary secretions and bronchitis. The second bacterium was *Nocardia cummidelen* which is present in the soil and could lead to infection in patients with immunodeficiency. In addition, *N. asteroides*, *Nocardia otitidiscaviarum*, and *Nocardia ignorata* had been isolated from many infections, especially pulmonary and ulcerative ones. Many researchers isolate *Nocardia* from soil and environment for biotechnology purposes; however, for prevention of hospital-acquired infections, there are few studies on this bacterium in green spaces and gardens around the buildings of cancer and transplant centres where the risk of infections is higher than in other centres. This species carries a threat to patients, in particular, to immunocompromised patients.

In 2017, Rahdar *et al.* 90 environmental samples were collected from dust, soil and water resources of 30 hospitals in the city of Isfahan. In their study, using phenotypic method and PCR based on 16S rRNA sequencing, a total of 25 *Nocardia* strains were isolated with the most frequent species as *N. cyriacigeorgica* (24%)[15]. In the Rahdar *et al.* Study, soil, dust, and wastewater of hospitals were used; while in the present study, the soil was sampled. We found that *N. cyriacigeorgica* was the predominant species with a high prevalence especially in individuals with immunodeficiency and transplant recipients. Most of the researches mainly focus on the biodiversity of these bacteria and the production aspects of secondary metabolites. phenotypic methods which include the macroscopic examination, microscopic assessments and other standard biochemical assays , and culturing in selected media are regarded as reference methods in *Nocardia* diagnosis, but these methods cannot be performed without access to suitable isolates of *Nocardia*. Besides, due to long incubation and slow-paced bacterial growth, the samples are likely to be contaminated by other environmental saprophytes during incubation. Therefore, molecular methods could provide more precise diagnosis of *Nocardia* species. The diagnosis of *Nocardia* is important for epidemiologic studies and the determination of the geographical distribution. Molecular methods such as PCR and 16S rRNA sequencing have higher specificity and sensitivity compared with phenotypic and microscopic methods and can make it possible to examine all known species of *Nocardia*.

In this study, a diversity of opportunistic species of the genus of *Nocardia* were studied by phenotypic, biochemical, and molecular methods from the green spaces and gardens surrounding transplantation and cancer centers of hospitals in the city of Tehran, Iran. The identification can help physicians to implement treatments and is important for healthcare centers, such as transplantation and cancer centers.

## Conflict of interest statement

The authors report no conflict of interest.

## Founding project

This study was part of a PhD thesis supported by Tehran University of Medical Science (Grant No: 34468270396).

## Authors' contribution

All authors were involved in the study design, data collection, concept, definition of intellectual content, literature search, experimental studies, data acquisition, data analysis, manuscript preparation, manuscript editing, and manuscript review.

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