Original Article

# Phenotypic characterization of *Nocardia* spp. isolated from Iran soil microflora

*Shadi Habibnia, Masoumeh Rasouli Nasab, Parvin Heidarieh[1], Mehdi Fatahi Bafghi, Mohammad Reza Pourmand, Seyyed Saeed Eshraghi*

Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, [1]Department of Bacteriology and Virology, School of Medicine, Alborz University of medical Sciences, Karaj, Iran

**Address for correspondence:**
Prof. Seyyed Saeed Eshraghi,
Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Qods St., Poursina St., Tehran, Iran.
E-mail: eshraghs@tums.ac.ir

## ABSTRACT

**Aims:** The present study was conducted to identify *Nocardia* spp. from Iran soil by various phenotypic tests.

**Materials and Methods:** A total of 300 soil samples were collected of five different geographical regions in Iran. *Nocardia* isolation was performed by paraffin baiting technique. The colonies that were similar to be *Nocardia* spp. were stained with Gram, partially acid fast and acid-fast. Phenotypic tests were used for identification of *Nocardia* spp.

**Results:** After analysis of phenotypic tests, the identified species are as follow: *Nocardia asteroides* (49.12%), *Nocardia cyriacigeorgica* (24.56%), *Nocardia otitidiscaviarum* (38.6%), *N. asteroides* complex (19.29%), *Nocardia africana* (3.5%), and *Nocardia pseudobrasiliensis*, *Nocardia coubleae*, *Nocardia ignorata* (each species; 1.75%). In this study, some of isolates (8.77%) remained undetected.

**Conclusion:** Due to great number of *Nocardia* spp., high similarities among biochemical characteristics of species and the variability of some these characters, a wide range of biochemical tests should be used to identify *Nocardia* spp. to gain more accurate results.

**Key words:** Iran, *Nocardia*, paraffin baiting technique, phenotypic tests

## INTRODUCTION

*Nocardia* are filamentous Gram-positive bacilli, aerobic, and partially acid-fast. The genus *Nocardia* is caused opportunity infections in immunosuppressive disorder, AIDS, cancer, diabetes and organ transplantation. They cause infections in the respiratory and extra respiratory tract, skin and brain. Pulmonary nocardiosis frequently is acquired through inhalation of aerosols containing the bacteria.[1-3] *Nocardia* spp. is soil saprophytic that can play an important role in the turnover of soil organic materials. This bacterium is significant organism in industries, agricultural, and biotechnology. In recent years, classification of the genus *Nocardia* is changed. Until date, 103 species of bacteria have been identified which some of them have been known as *Nocardia asteroides* complex and *Nocardia nova* complex.[4]

Identification of *Nocardia* spp. from each region is very important because the distribution of these species due to

Access this article online

Quick Response Code:

Website: www.ijehe.org

DOI: 10.4103/2277-9183.158388



This article may be cited as:
Habibnia S, Nasab MR, Heidarieh P, Bafghi MF, Pourmand MR, Eshraghi SS. Phenotypic characterization of *Nocardia* spp. isolated from Iran soil microflora. Int J Env Health Eng 2015;4:20.

climatic variations is different in each region. A few literatures have been reported identification of *Nocardia* spp. in Iran soil.[5,6] The aim of this study is significant for two reasons: First, the investigation of *Nocardia* recognition in different geographical regions is important; second, the utilization of various phenotypic tests can help us to identify more accurate *Nocardia* spp. This study was not to evaluate the epidemiology, but isolation and evaluation of *Nocardia* spp. from all regions in Iran.[5] According to the environmental condition, each region has special species of *Nocardia*, and, on the other hand, treatment is different for each species. Therefore, identification species any area is important to follow-up treatment.[7]

There are several methods to isolate *Nocardia* from soil such as paraffin baiting method, humic acid vitamin B agar and paraffin agar and sucrose gradient centrifugation. Based on recent studies, paraffin baiting technique is more effective than other methods for *Nocardia* isolation of soil because it is cost-effective, and also this method prevents the growth of other organisms.[2,8-11] Hence, we investigated *Nocardia* spp. in Iran soil by various phenotypic tests.


**Figure 1:** *Nocardia* isolates of geographical regions from Iran

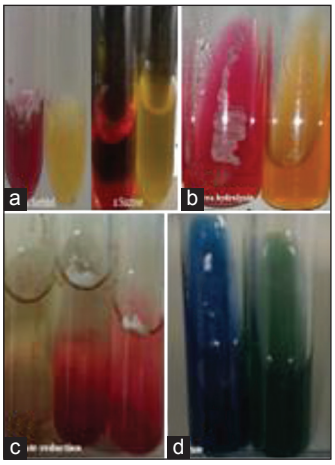


**Figure 3:** (a) Production acid of carbohydrates; (b) urea hydrolysis; (c) nitrate reduction; (d) Utilization of citrate

## MATERIALS AND METHODS

Soil samples were randomly collected from 4 cm deep within 15 months (from March 2011 until July 2013). In this study, 300 samples were collected from various soils from five geographical regions (North, West, East, South, and central) of Iran [Figure 1]. The samples were collected in sterile plates and were transferred to actinomycetes lab (Tehran University of Medical Sciences, School of Public Health) within 24-48 h.

### Isolation method

In this study, isolation of *Nocardia* performed by paraffin baiting technique. A total of 1 g of soil mixed into a sterile tube in 10 ml of distilled water and shacked for 5 min. About 1 ml of the supernatant is added to the carbon-free broth tube with paraffin sticks and incubated at 35°C for 20-30 days (every 3 days, the paraffin sticks were examined). The white colonies similar to *Nocardia* on paraffin stick were cultured on nutrient agar and sabouraud dextrose agar (contain cycloheximide, Sigma-Aldrich, USA) for purification.[12]

### Phenotypic tests

The colonies that suspected to *Nocardia* spp. were stained with Gram, partially acid-fast and acid-fast. Lysozyme broth was used for the identification of the genus *Nocardia*. Colonies were investigated with a stereo microscope. *Nocardia* spp. identification was according to biochemical tests that are including: Growth in lysozyme

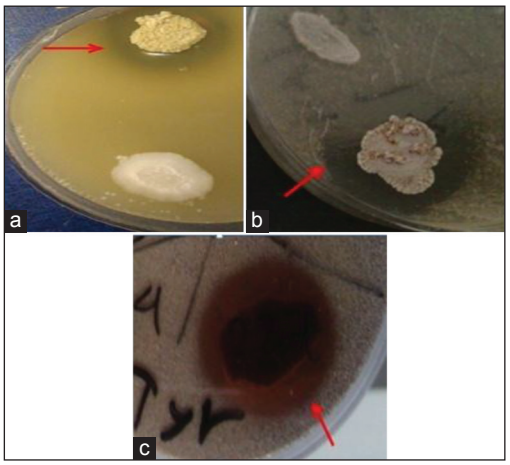


**Figure 2:** (a) Hydrolysis of casein; (b) hypoxanthine; (c) tyrosine

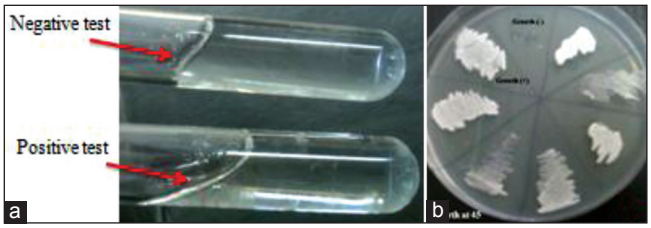


**Figure 4:** (a) Hydrolysis of gelatine, (b) growth at 45°C

broth, hydrolysis of amino acids such as hypoxanthine, xanthine, tyrosine, casein, gelatin and urea, production of nitrate reductase (Sigma-Aldrich, USA), Growth at 45°C, producing acid from carbohydrates such as glucose, maltose, lactose, galactose, salicine, xylose, raffinose, arabinose, rhamnose, sorbitol, and sucrose (Merck, Germany).[1]

## RESULTS

Among 300 soil samples cultured with paraffin baiting method, 65 isolates were suspected to be similar colonies to *Nocardia* spp. (white, cream-colored and chalky) (21.66%). Colonies stained and eight isolates eliminated because they were contaminated by fungal and *Bacillus* spp. 57 isolates (19%) were identified with initial tests.

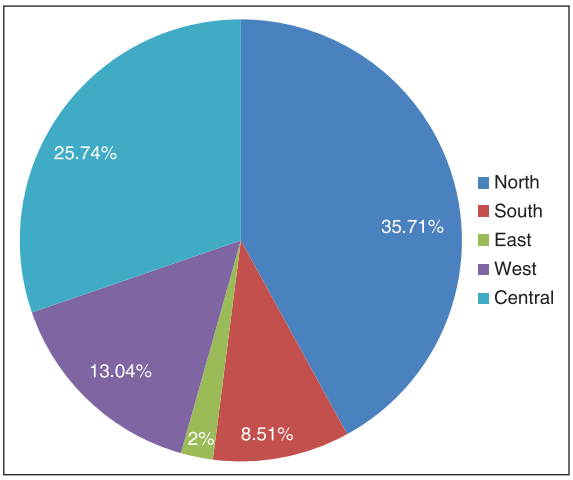


**Figure 5:** Percentage of *Nocardia* isolates in Iran soil

Colonies were positive with Gram and partially acid-fast and they were negative for acid-fast. The genus *Nocardia* was confirmed by the growth in lysozyme broth. Then, various biochemical tests were performed for these strains of *Nocardia* [Figures 2-4]. The analysis of phenotypic tests were identified that 19 isolates of *N. asteroides* (49.12%), 14 isolates of *Nocardia cyriacigeorgica* (24.56%), 3 isolates of *Nocardia otitidiscaviarum* (38.6%), 11 isolates of *N. asteroides* complex (19.29%), 2 isolates of *Nocardia africana* (3.5%), 1 isolate of *Nocardia pseudobrasiliensis*, *Nocardia coubleae*, and *Nocardia ignorata* (each species; 1.75%). In our study, five isolates remained (8.77%) undetected. Most isolates belonged to the *N. asteroides* complex [Table 1].

## DISCUSSION

*Nocardia* is soil saprophytes that widespread all over the world. Thus, it can potentially transmitted to human with inhalation in contaminated soil or skin injury. *Nocardia* isolation can vary in different geographical areas based on soil type.[12] We recognized of *Nocardia* in different regions by various phenotypic tests.

Aghamirian and Ghiasian in 2006-2007, studied 300 soil samples of different areas of Qazvin, Iran. Samples were cultured on brain-heart infusion agar and Sabouraud's dextrose agar contains cycloheximide. Overall, were isolated *N. asteroides* (15.7%), *N. otitidiscaviarum* (9.4%), and *Nocardia brasiliensis* (7.3%) that were cultured on brain-heart infusion agar and Sabouraud's dextrose agar contain cycloheximide.[5] Kachuei *et al*. isolated *Nocardia* spp. of soil in different regions from Isfahan province

**Table 1: Physiological characteristics of *Nocardia* isolates**

| Number of isolate | Lysozyme | Casein | Tyrosine | Hypoxanthine | Xanthine | Nitrate reduction | Urea hydrolysis | Growth at 45 | Gelatin | Citrate | a-L-rhamnose |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1, 2, 3, 5, 6, 15, 22, 27, 31, 35, 20, 50, 55, 56, 57 | + | − | − | − | − | + | + | − | V | + | − |
| 11, 19, 21, 23, 24, 38, 42, 51, 53, 9, 4, 26, 40, 48 | + | − | − | − | − | − | + | − | + | − | − |
| 8, 39, 43 | + | − | − | + | W | + | + | + | − | − | − |
| 10, 41, 44, 45, 49, 52, 54 | + | − | − | − | − | − | W | W | + | + | − |
| 12, 28 | + | + | − | − | − | + | + | − | + | + | − |
| 46 | + | − | − | − | − | + | − | + | − | − | − |
| 25 | + | + | + | + | − | − | + | − | − | + | − |
| 29 | + | + | − | − | − | + | + | + | − | − | − |
| 7 | + | − | − | + | − | − | + | − | − | + | − |
| 13 | + | − | − | − | − | + | + | − | − | + | − |
| 34 | + | − | + | − | + | − | + | − | − | + | − |
| 36 | + | − | − | − | − | + | + | − | − | + | − |
| 37 | + | − | − | + | − | − | + | − | − | + | − |

Continued

**Table 1: (Continued)**

| D (+) −sorbitol | D (+) −xylose | Raffinose | Sucrose | D-lactose | L-arabinose | Glucose | Maltose | D-galactose | Salicin | Isolates are identified as* |
|---|---|---|---|---|---|---|---|---|---|---|
| − | − | − | − | V | − | V | V | − | − | *N. asteroides* |
| − | − | V | V | − | − | V | V | − | − | *N. cyriageorgica* |
| − | − | V | − | − | − | V | V | − | − | *N. otitidiscaviarum* |
| − | − | − | − | − | − | − | − | − | − | *N. asteroids* complex |
| − | − | − | − | | − | + | − | − | − | *N. africana* |
| − | − | − | + | | − | − | + | − | − | *N. coubleae* |
| + | + | − | − | − | + | + | + | W | − | *N. psudobrasiliensis* |
| − | − | − | − | − | − | + | − | − | − | *N. ignorata* |
| − | − | − | + | − | − | − | + | − | − | *N.* spp. |
| − | − | + | − | + | + | − | − | − | − | *N.* spp. |
| − | + | − | − | + | − | − | − | + | − | *N.* spp. |
| − | − | − | − | + | + | − | − | − | − | *N.* spp. |
| − | − | − | − | + | + | − | + | − | − | *N.* spp. |

+: Positive, −: Negative, W: Weakly, V: Variable, *N.*: *Nocardia*, *Results are consistent with information obtained through the phenotypic characteristics of reference strains, and studies of the website DSMZ,[4] Yasin (2000),[17] Kiska (2002),[18] Yamamura (2003),[11] and Brown-Elliot (2006) *et al*.[1]

in the center of Iran. Identification of species was done with kanamycin and conventional biochemical tests such as tyrosine, casein, hypoxanthine, xanthine, starch and gelatin. From 153 isolates, *N. asteroides* complex (45.5%), *Nocardia brasiliensis* (24.7%), *N. otitidiscaviarum* (2.2%), *Nocardia transvalensis* (1.1%), *Nocardiopsis dassonvillei*, *Actinomadura madura* (each one 1.7%) were identified. Also 23.0% of total remained undetected.[6] Another study by Wauters *et al.* from clinical samples with phenotypic methods in 2005 reported *Nocardia farcinica* 44%, *Nocardia nova* 22%, and *N. cyriacigeorgica* 15%.[13]

According to the results obtained in this study using a range of phenotypic tests, more species of *Nocardia* was identified. In addition to identification of *N. asteroides*, another species, for example, *N. africana*, *N. coubleae*, *N. ignorata*, and *Nocardia psudobrasiliansis* were determined. Wide range of biochemical tests for identification of *Nocardia* spp. from clinical samples were used. This investigate is the first study were gathered soil samples in different climatic regions of Iran to isolation species of *Nocardia*. Most of *Nocardia* spp. were isolated from north of Iran [Figure 5]. In the present study, the highest percentage of *Nocardia* spp. were isolated from forest and garden zones. Also, percent of *Nocardia* isolates in different areas is: Gardens (15.78%), cultivated lands (35%), parks (20%), forest (31.42%), desert regions (15.38%), green spaces and boulevards (14.28%), greenhouse (17.24%), and sludge (7.69%). The positive samples were not isolated from coastal areas [Table 2]. Therefore, probably conditions for the growth of *Nocardia* are favorable due to the presence of organic matter and nutrients in different soil, suitable moisture, and pH.

Due to the increasing number of new species in the genus *Nocardia* and limited biochemical tests so cannot identify precisely and reliably. The use of a wide range of biochemical tests can be helpful in more accurate identification of species. In the present study, we used various phenotypic tests to identify of *Nocardia* spp.

**Table 2: Rate of *Nocardia* isolated from different regions of Iran**

| Regions of Iran | Areas of sample collection | The number of samples collected | The number of positive samples |
|---|---|---|---|
| South | Gardens | 8 | 0 |
| | Desert regions | 13 | 2 |
| | Green spaces and boulevards | 12 | 1 |
| | Greenhouse | 9 | 1 |
| | Coastal | 5 | 0 |
| East | Park | 16 | 0 |
| | Green spaces and boulevards | 10 | 1 |
| | Cultivated land | 6 | 0 |
| | Sludge | 4 | 0 |
| North | Gardens | 15 | 6 |
| | Forest | 22 | 9 |
| | Green spaces and boulevards | 8 | 2 |
| | Coastal | 8 | 0 |
| West | Cultivated land | 4 | 2 |
| | Parks | 8 | 1 |
| | Forest | 13 | 2 |
| | Green spaces and boulevards | 5 | 0 |
| | Greenhouse | 6 | 0 |
| Central | Garden | 34 | 3 |
| | Cultivated land | 16 | 5 |
| | Parks | 26 | 9 |
| | Green spaces and boulevards | 21 | 4 |
| | Greenhouse | 8 | 4 |
| | Sludge | 9 | 1 |

## CONCLUSION

Due to great number of *Nocardia* spp., high similarities among biochemical characteristics of species and the variability of some of these characteristics, a wide range of biochemical tests should be used to identify *Nocardia* spp. to gain more accurate results. We recommend usage of molecular methods such as polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism to confirm phenotypic tests results.[14-16]

## ACKNOWLEDGMENT

This study was supported by Tehran University of Medical Sciences, Deputy of Research.

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**Source of Support:** Tehran University of Medical Sciences, **Conflict of Interest:** None declared.