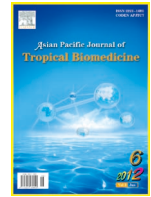




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## Diversity and frequency of *Nocardia* spp. in the soil of Isfahan province, Iran

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## ABSTRACT

**Objective:** To isolate and identify *Nocardia* spp. from soil in different regions of Isfahan province in the center of Iran. **Methods:** This study was conducted in 32 districts (16 cities and 16 villages) in Isfahan province during two years. A total of 800 soil samples from these regions were studied by using kanamycin. The isolated *Nocardia* species were examined by gram and acid-fast staining and were identified biochemically and morphologically. The frequency and distribution of *Nocardia* spp. were determined in relation to different factors such as soil pH and temperate climate. **Results:** From 153 (19.1%) *Nocardia* isolates identified, *Nocardia asteroides* (*N. asteroides*) complex (45.5%) and *Nocardia brasiliensis* (*N. brasiliensis*) (24.7%) were the most frequently isolated species, followed by *Nocardia otitidiscaviarum* (2.2%), *Nocardioopsis dassonvillei*, *Actinomadura actinomadura* (each 1.7%) and *Nocardia transvalensis* (1.1%) and also unknown spp. (23.0%). In this study, most species (54.4%) of *Nocardia*, especially *N. asteroides* complex were isolated from soils with pH: 7.01–8, whereas in pH: 8.01–9 more *N. brasiliensis* was isolated. The most *Nocardia* spp. was detected from regions with semi-nomadic and temperate climate (41.1%). **Conclusions:** *N. asteroides* complex is more prevalent in Isfahan province and soil can be a potential source of nocardiosis infections. It is to be considering that climate and soil pH are involved in the frequency and diversity of aerobic *Actinomycetes*.

### 1. Introduction

*Nocardia* species are aerobic *Actinomycetes*, gram-positive, non-spore, filamentous, branching, obligatory aerobic and relatively slow-growing bacterial[1]. So far, more than 50 species in the genus *Nocardia* are described among which *Nocardia asteroides* (*N. asteroides*) complex, *Nocardia brasiliensis* (*N. brasiliensis*), *Nocardia transvalensis* (*N. transvalensis*), *Nocardia otitidiscaviarum* (*N. otitidiscaviarum*), *Nocardia farcinica* and *Nocardia pseudobrasiliensis* are more pathogenic in humans[2]. These organisms are found in sludge, soil, contaminated soils water, plants, spoiled material and

plant and cause opportunistic infections in humans and animals[3–10].

Increase in the infections caused by these organisms, with different clinical forms of systemic and disseminated forms of nocardiosis in transplant recipients, tuberculosis, liver cirrhosis, cancer, diabetes, AIDS and patients under treatment with immunosuppressive drugs and broad spectrum antibiotics in the world, reveals more importance of isolation and identification of these agents[2,11–24]. Various studies have shown that identification and isolation of these agents in soil of different areas help to diagnose nocardiosis[1].

Aerobic *Actinomycetes* are found in soils in different parts of the world. However, according to various factors such as environment and ecological factors like temperature, humidity, vegetation zone, etc., their abundance is variable in different regions of the world. Nocardiosis is sporadic in Iran, but in other countries it is more prevalent. In USA, 500–1000 new case of nocardiosis was annually reported[1,3,4,25].

Due to the climate and vegetation diversity in Isfahan

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province, which is one of the major tourist cities in the world, the present study was carried out with the goals of isolation and identification of different species of *Nocardia* in different regions and investigating the effect of environmental factors such as soil pH and type of climate on the frequency of these microorganisms in soil.

## 2. Materials and methods

### 2.1. Study site

This study was done in Isfahan province (32°39'N, 51°40'E, with an area of 107 027 km<sup>2</sup>) in the center of Iran, during 2007–2008.

### 2.2. Sampling

A total of 800 soil samples were collected randomly from 800 different locations in 16 townships (including 16 cities and 16 villages) in Isfahan province. Fifty soil samples from each township (including 30 samples from central city and 20 samples from selected villages) were collected (Figure 1).



**Figure 1.** The map of soil sampling location in Isfahan province.

Sampling was carried out in winter and work on the samples lasted for 18 months. Sampling locations in cities included squares, boulevards, sidewalk, area offices, coastal and forest parks, rivers, waterfall and samples were collected in villages from corral livestock and domestic animals, nests, farm land, fruit gardens, garden houses, etc. Soil samples to the amount of 100–200 g for each sample were collected from superficial layer with depth not exceeding 2–5 cm. The samples were placed in sterile polyethylene bags, transported immediately to the laboratory and were stored at low temperature (4 °C) until tested. The pH of the samples was measured immediately in a 1:5 soil/deionized

water suspension (w/v) using a pH meter according to a previous study<sup>[26]</sup>.

Studied townships based on air temperature and humidity that in the sampling phase were measured. Considering the geographical situation, townships were divided into 3 groups including townships with cold and semi-wet climate, semi-nomadic climate and desert climate (Table 1).

**Table 1**

Separation of studied townships according to the type of climate.

Climate	Townships
Cold and semi-wet	Khansar, Semirom, Feraidan, Feraidoonshahr, Golpayegan, Natanz
Semi-nomadic	Borkharomeimeh, Shahreza, Flavarjan, Lenjan, Mobarekeh
Desert	Ardestan, Khomainishahr, Kashan, Nain, Najafabad

In this study, three methods were evaluated during a pre-test. Initially, the method of glass rods dipped in paraffin in 30 soil samples from Falavarjan city was used, but due to high bacterial contamination, it did not achieve satisfactory results and only two positive samples with bacterial contamination were found. Then, two soil samples positive by dilution method with chloramphenicol and tetracycline antibiotics were evaluated. In this method, no *Nocardia* spp. and other *Actinomycetes* were isolated and only bacterial contamination was observed. Then, to solve this problem, the third method that used the medium containing kanamycin on these two samples was utilized in which satisfactory results were obtained.

### 2.3. Isolation method

For preparing suspensions, 3–5 g of soil samples were added to tubes containing 10 mL sterile saline and then tubes were shaken for 3 min. Then the suspension was incubated for 15 min and thereafter, 2–5 mL of the supernatant solution was transferred to another sterile tube by sterile pipette.

Streptomycin, chloramphenicol and antibiotic solutions (2 mg/mL) were added to half the volume of supernatant solution and the mixture was incubated for half an hour after being stirred up. Then after being shaken again, one drop (0.05 mL) of a brain-heart infusion (BHI) agar medium (Merck, Germany), containing cycloheximide (0.5 g/L) and kanamycin (25 mg/L) was added to the tube immediately, and then the mixture was incubated at 37 °C for 2 weeks<sup>[14]</sup>. During this period, the wrinkled colonies of red, orange, yellow, and white to cream colors suspicious of *Nocardia* and other aerobic *Actinomycetes* were considered and cover slip-buried methods were used. If delicate and branched filaments which are the characteristics of *Actinomycetes* were seen, they were isolated by Sabouraud dextrose agar medium in tubes. Then by using Kinyoun staining, conventional biochemical tests and physiological criteria

such as the capability to degrade the organic compounds such as tyrosine, casein, hypoxanthine, xanthine and starch as substrates, as well as growth in 4% gelatin medium, were studied in order to reach a possible classification to the species level[1].

### 3. Results

Among the 800 soil samples, 153 (19.1%) were positive for *Nocardia* spp. and other aerobic *Actinomycetes*. A total of 178 isolates of *Nocardia* spp. and other aerobic *Actinomycetes* were recognized. They belonged to 6 species of 3 genus as follows: *N. asteroides* complex (45.5%), *N. brasiliensis* (24.7%), *N. otitidiscaviarum* (2.2%), *Nocardiopsis dassonvieli* (*N. dassonvieli*) and *Actinomadura madurae* (*A. madurae*) (1.7%), *N. transvalensis* (1.1%) and unknown spp. (23.0%) (Table 2).

In this study, in most cities, *N. asteroides* complex was the dominant species. Moreover, more *Nocardia* spp. and other aerobic *Actinomycetes* were isolated from urban areas than rural regions (Figure 2).

**Table 2**

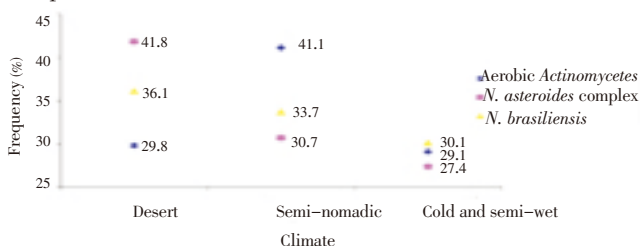
Frequency of *Nocardia* spp. and other aerobic *Actinomycetes* isolated from soils of 16 townships in the province of Isfahan, Iran.

Parameters	Townships																Total	% Frquency
	Ard	Bor	Fer	Fla	Fsh	Gol	Kas	Kha	Kho	Len	Mob	Nai	Naj	Nat	Sem	Sha		
No. of samples examined	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	50	800	
Positive samples	4 (8)	2 (4)	13 (26)	21 (42)	6 (12)	3 (6)	7 (14)	10 (20)	8 (16)	16 (32)	9 (18)	13 (26)	12 (24)	9 (18)	8 (16)	12 (24)	153 (19.1)	
Species isolated																		
<i>N. asteroides</i> complex	3	0	7	10	4	1	3	6	6	8	7	4	5	6	4	7	81	45.5
<i>N. brasiliensis</i>	1	1	3	6	2	2	3	1	2	5	0	5	4	4	3	2	44	24.7
<i>N. otitidiscaviarum</i>	0	1	0	0	1	0	0	0	0	2	0	0	0	0	0	0	4	2.2
<i>N. transvalensis</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	2	1.1
<i>A. madurae</i>	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	3	1.7
<i>N. dassonvieli</i>	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	1	3	1.7
Unknown spp.	2	0	4	8	2	0	2	4	1	4	3	3	3	1	1	3	41	23
Total	6	2	17	24	9	3	8	11	9	19	10	13	14	11	8	14	178	100

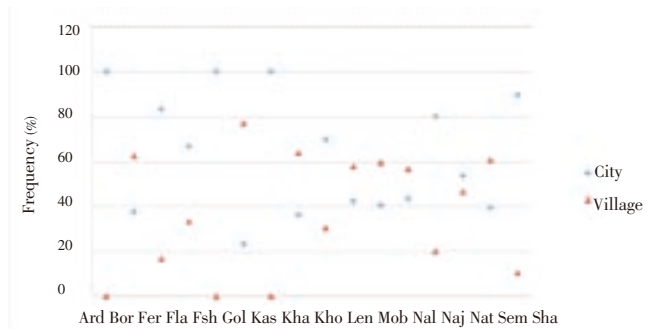
Ard: Ardestan; Bor: Borkharomeimea; Fer: Feraidan; Fla: Flavarjan; Fsh: Feraidoonshahr; Gol: Golpayegan; Kas: Kashan; Kha: Khansar; Kho: Khomainishahr; Len: Lenjan; Mob: Mobarekea; Nai: Naiin; Naj: Najafabad; Nat: Natanz; Sem: Semirom; Sha: Shahreza.

this type of climate (Figure 3). In addition, *N. brasiliensis* (36.1%) were most detected from regions with desert climate (Figure 3).

Figure 4 showed the distribution of the *Nocardia* spp. and other aerobic *Actinomycetes* in relation to soil pH. We found 54.5% *Nocardia* spp. and other aerobic *Actinomycetes* from the soil samples with pH: 7.01–8 and 45.5% from pH: 8.01–9. We did not find aerobic *Actinomycetes* from the soil samples with pH: 6–7.



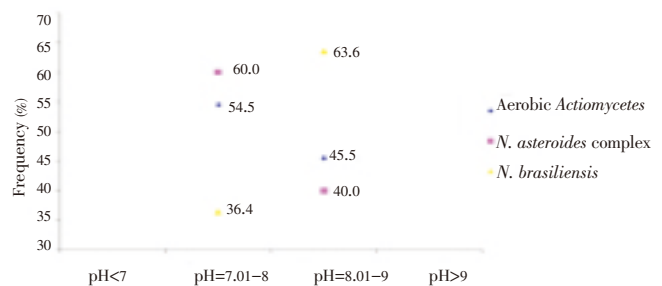
**Figure 3.** Frequency of *Nocardia* spp. and aerobic *Actinomycetes* isolated from soils of 16 townships in the province of Isfahan, Iran at different climate.



**Figure 2.** Frequency of *Nocardia* spp. and other aerobic *Actinomycetes* isolated from soils of urban and rural regions from 16 townships in the province of Isfahan, Iran.

Ard: Ardestan; Bor: Borkharomeimea; Fer: Feraidan; Fla: Flavarjan; Fsh: Feraidoonshahr; Gol: Golpayegan; Kas: Kashan; Kha: Khansar; Kho: Khomainishahr; Len: Lenjan; Mob: Mobarekea; Nai: Naiin; Naj: Najafabad; Nat: Natanz; Sem: Semirom; Sha: Shahreza.

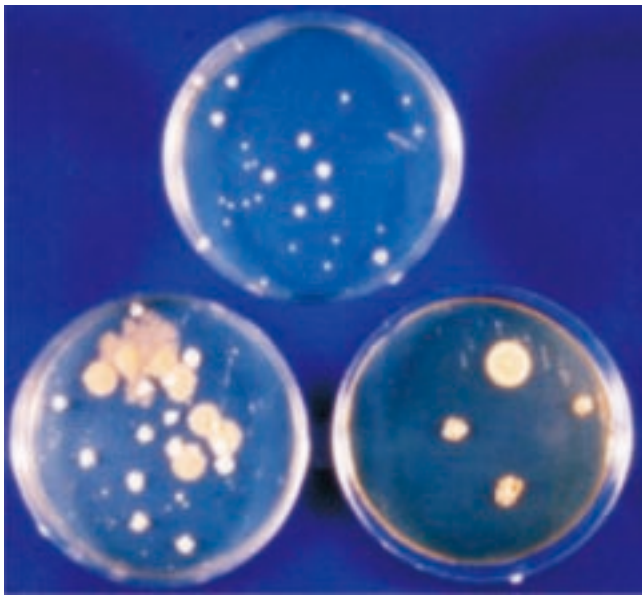
In the present study, most *Nocardia* spp. and other aerobic *Actinomycetes* were detected from regions with semi-nomadic and temperate climate (41.1%). Also most *N. asteroides* complexes (30.7%) were detected from regions with



**Figure 4.** Frequency of *Nocardia* spp. and aerobic *Actinomycetes* isolated from soils of 16 townships in the province Isfahan, Iran at pH different values.

In the present study, most *N. asteroides* complex species (60.0%) were isolated from the soil samples with pH: 7.01–8. In contrast, most *N. brasiliensis* species (63.6%) were isolated from the soil samples with pH: 8.01–9 (Figure 4).

Figure 5 showed isolated *Nocardia* spp. from BHI agar medium containing cycloheximide (0.5 g/L) and kanamycin (25 mg/L), which was incubated at 37 °C for 7–10 day.



**Figure 5.** Isolated *Nocardia* spp. from BHI agar medium containing cycloheximide (0.5 g/L) and kanamycin (25 mg/L).

#### 4. Discussion

In this study, isolation of different species of *Nocardia* spp. from soil in different regions of Isfahan, Iran and the effect of environmental factors such as soil pH and types of climate on frequency of these bacteria in soil were examined. In the present study, BHI agar medium containing two antibiotics (kanamycin and cycloheximide) were used and it was shown that the growth of *Nocardia* and *Actinomadura* was stimulated by kanamycin and in most cases in positive samples, only one colony or purified species of *Nocardia* was observed without microbial contamination. For the first time, Vetlugina *et al* used kanamycin for the isolation of *Nocardia* spp. from soil<sup>[14]</sup>. In this research, paraffin bait method was not applied because of bacterial contamination and some species of *Nocardia* and *Actinomycetes* do not grow on paraffin substrate.

In our study, *Nocardia* prevalence in soil was 16.4%. The frequency of pathogenic *Nocardia* species acquired from soil samples in different regions of the world has been reported to be 5%–50%<sup>[27–30]</sup>. In other survey in Iran the isolation rate of *Nocardia* from soil was reported to be 40.6%<sup>[25]</sup>. Differences observed in these studies could be due to type of studied soil and methods used<sup>[29]</sup>.

In this study, the dominant species was *N. asteroides* complex. These results are similar with studies of Khan *et al*<sup>[28]</sup>, Van Gelderen *et al*<sup>[30]</sup>, and Ajello *et al*<sup>[31]</sup>.

Studies in Iran show that like other parts of the world, the dominant agent of nocardiosis is *N. asteroides* complex. Nocardiosis in Iran and other parts of the world in clinical

forms of pulmonary, sinusitis and mycetoma has been reported<sup>[32–39]</sup>. Also, like the study performed by Stapleton *et al*, in this study it was found that the rate of isolation of *Nocardia* spp. in semi-desert climate to colder regions is higher<sup>[40]</sup>.

In this study, most species (54.4%) of *Nocardia*, especially *N. asteroides* complex was isolated from soils with pH: 7.01–8, whereas in pH: 8.01–9 more *N. brasiliensis* was isolated. It is to be considering that climate and soil pH are involved in the frequency and diversity of aerobic *Actinomycetes*. There is no previous report about relationship between pH and diversity of *Nocardia* in soil.

In this study, *Streptomyces* were not isolated, which was probably due to the following reasons: 1) according to Rippon<sup>[41]</sup>, species of *Streptomyces* have been observed in soils with pH less than 6.5, whereas in this study, about 90% of soil samples were with pH>7; 2) another possible reason can be the sensitivity of *Streptomyces* to the amount of kanamycin used.

In general, soil pH and type of climate were probably involved in the rate of *Nocardia* spp. isolated from soil. Also kanamycin in culture medium is useful in isolation of *Nocardia*. It is also necessary to mention that this technique was used for the isolation of aerobic *Actinomycetes* from soil samples for the first time in Iran.

#### Conflict of interest statement

We declare that we have no conflict of interest.

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