Isolation and Characterization of Medically Important Aerobic Actinomycetes in Soil of Iran (2006 - 2007)

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Abstract: The aerobic *actinomycetes* are a large group of soil-inhabiting bacteria that occur worldwide. Some of them are the main cause of two important diseases, nocardiosis and actinomycetoma. To identify the prevalence and geographic distribution of aerobic actinomycetes in soil of Qazvin province, a study was carried out during 2006-2007. In this study, the incidence and diversity of medically important aerobic actinomycetes was determined in 300 soil samples of different parts of Qazvin. The suspensions of superficial soil samples were prepared by adding of normal saline, streptomycin and chloramphenicol and the supernatants were cultured on brain-heart infusion agar and Sabouraud's dextrose agar contain cycloheximide. The isolated microorganisms were examined by Gram and acid-fast stains and were identified biochemically and morphologically. Of 96 aerobic actinomycetes isolates identified, *Actinomadura madurae* and *Streptomyces somaliensis* were the most frequently isolated species each representing 19.8% of isolates, followed by *Nocardia asteroides* (15.6%), *N. otitidiscaviarum* (9.4%), *N. brasiliensis* (7.3%), *A. peletieri*, *S. griseus*, and *Nocardia* spp. (each 5.2%), and *N. transvalensis*, *Nocardiopsis dassonvillei*, *Actinomadura* spp. and *Streptomyces* spp. (each 3.1%). To the best of our knowledge, this is the first report on epidemiological investigation of medically important aerobic actinomycetes in soil samples from Iran. In recent years, mycetoma and nocardiosis have been increasingly reported in Iran. The results showed that medically important actinomycetes occur in the environment of Iran and soil could be potential source of actinomycotic infections.

Key Words: Aerobic actinomycetes, soil, Qazvin, Iran.

INTRODUCTION

Actinomycetes are found worldwide, maintaining a saprophytic existence in soil and other natural habitats. Soil is a major reservoir for retention of bacteria and fungi and wind can transmit these agents in companion with dust and dirt into lung or skin and produce diseases like nocardiosis, sporotrichosis, chromomycosis, mycetoma etc [1].

The aerobic actinomycetes are a various group of Gram-positive, branching, filamentous, obligately aerobic and relatively slow-growing bacteria. Actinomycetes are considered as Spore-forming bacteria with indication of health problems [1]. It is widely recognized that aerobic actinomycetes abundantly found in soil and cause opportunistic infections in human and animals and even cause death in some occasions. It has been known that *Streptomyces* species can produce secondary metabolites [2]. Toxic metabolites isolated from *Streptomyces* and *Nocardiopsis* isolates have shown to be mitochondriotoxic in spermatozoa [3]. It is recognized that some isolates of *Streptomyces griseus* produce valinomycin, a toxin that cause mitochondrial swelling, activity loss, and apoptosis in human natural killer cells [4].

Streptomyces species may be implicated in respiratory disorders of individuals living in moldy houses [5]. Increase in the infections by aerobic actinomycetes, specially *Nocardia* genus and diverse array of clinical illnesses specially the systemic and disseminated forms of nocardiosis in cancer, tuberculosis, diabetes, AIDS and patients under treatment with immunosuppressive drugs and broad spectrum antibiotics in the world reveal importance of detection of these agents more than ever [6]. Actinomycetes present in soil like *Streptomyces somaliensis*, *Nocardia brasiliensis*, *N. asteroides*, *Actinomadura peletieri*, *A. madurae* also can be inoculated in the skin in a companion with dust and dirt and may cause actinomycetoma [6, 7]. Mycetoma and nocardiosis are sporadically spread in Iran and treatment of mycetoma essentially depends on the etiologic agent involved. In order to determine the identities and diversity of soil actinomycetes in Qazvin district of Iran, a descriptive analytical study conducted to find out if soils are potential sources of actinomycetes infections. To the best of our knowledge, this is the first report on epidemiological investigation of distribution of aerobic actinomycete populations in soil samples from Iran.

MATERIALS AND METHODOLOGY

Qazvin is a province situated on the northern west of capital, Tehran. The average summer temperature ranges from 25 to 35°C and humidity is up to 50%. Qazvin city covering an area of 50 sq. kms and has been divided into 25 regions (Fig. **1**).

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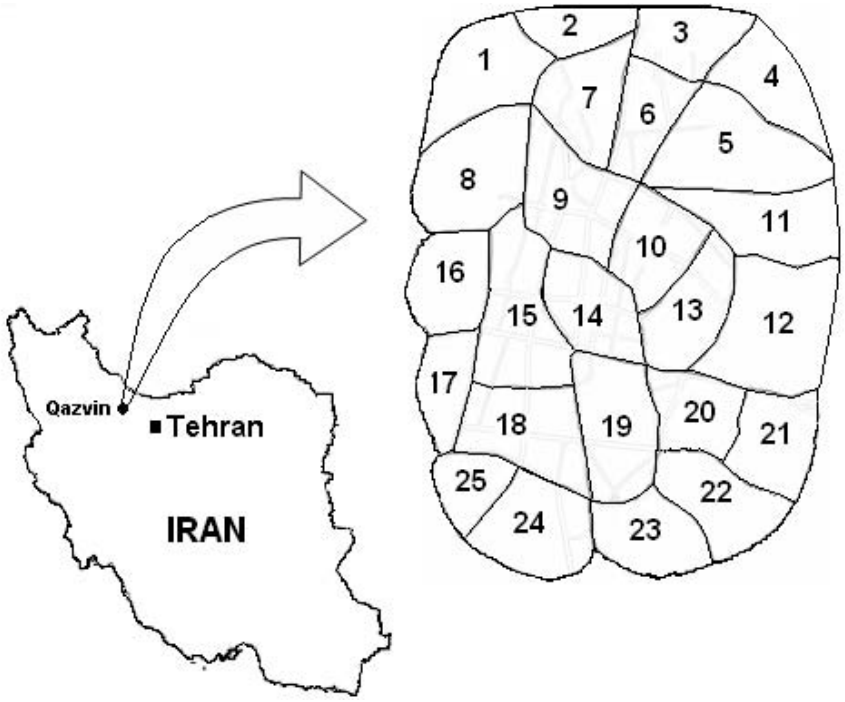

Fig. (1). Map of Iran, showing relative position of Qazvin with capital and 25 regions, from which samples were collected.

Over the period of the study (March 2006 to March 2007), total 300 soil samples were collected from 25 locations of the city, that is, 12 samples were randomly taken from 350 to 400 meters interval in each region. After removing the surface loose litter layer (approximately top 4 cm), the soil samples were taken from a depth of 5 to 10 centimeters of the superficial layers in each location. The soil type was sandy-clay in all over the city. Soil samples were numbered and put in a sterilized paper sack, transferred to the Mycology Laboratory of the School of Medicine, Qazvin University of Medical Sciences, where they were homogenized and spread in trays to be cleaned of leaves, roots, small stems, etc. These samples were sieved (2 mm mesh) and air dried at 25 °C for 7 days. Then five grams of each soil sample were added to a test tube containing 10 ml of sterile physiologic saline (0.9% NaCl) and mixed for 3 minutes. The suspension was incubated for 15 minutes and thereafter, 3 ml of the supernatant solution was transferred to another sterile test tube and 1.5 ml of antibiotic solutions of streptomycin and chloramphenicol (0.2 mg/ml) was added and incubated for half an hour after being stirred up. This solution was then shaked again and one drop (0.05 ml) was added to a Sabouraud's dextrose agar medium (BioMeriéux, Marcy-1, Etoil, France), containing 0.5 g/l of cycloheximide and another drop to brain-heart infusion agar containing 0.5 g/l of cycloheximide immediately, and incubated at 35°C for 2-3 weeks. Representative colonies were selected and streaked on new plates of aforementioned culture media. Actinomycetes on the plates were identified as colored, dried, rough, with irregular/regular margin, generally convex colony. The plates were kept in a 35°C incubator and examined for up to 2 or 3 weeks for possible growing, although the majority was detected within the first several days of incubation. During this period, the wrinkled colonies of yellow, red, orange, and white to cream colors suspicious of actinomycetes were considered and in order to purification of actinomycetes, the streak plate method were used. After isolation of the pure colonies based on their colonial morphology, colour of hyphae, color of aerial mycelia, they were individually plated on the same agar media.

Morphological examination of the actinomycetes was done by using cellophane tape and cover slip-buried methods. The mycelium structure, color and arrangement of arthrospore on the mycelium were examined using light microscopy under oil immersion (1,000 x). Since a single method could not identify all actinomycete isolates to the species level; therefore, a combination of methods was necessary. If branched and delicate filaments specific of actinomycetes were seen, they were isolated and examined for partial acid-fastness by staining with the carbolfuchsin modified acid-fast stain with a weak (0.5-1%) sulfuric acid decolorizing solution. Moreover, conventional biochemical tests and physiological criteria such as the capability to degrade the organic compounds such as casein, tyrosine, xanthine, hypoxanthine and starch as substrates, the utilization of urea and different carbon sources as well as growth in 4% gelatin medium were studied in order to reach a possible classification to the species level. Gelatin hydrolysis was determined by stabbing a loopful of organism about one cm into a nutrient gelatin tube (BBL), which were incubated at 35°C in air for up to 7 days. Detection of hydrolysis was accomplished by placing the tubes at 4°C for 15 min and then tilting each tube at a 45° angle. The test was considered positive if the gelatin was liquefied. For urea hydrolysis, a heavy loopful of bacteria was suspended in 0.5 ml of a medium containing 1 g urea and 1 ml of a cresol red solution (0.5% [wt/vol]) in 100 ml phosphate buffer (0.005 M, pH 6). Tubes were incubated at 35°C and checked after 1 and 2 days for a pink color shift representing a positive reaction.

RESULTS

Of 300 soil samples studied, a total of 96 actinomycete isolates, representing 9 species, were diagnosed including

Nocardia asteroides, *N. otitidiscaviarum* (Formerly *N. caviae*), *N. brasiliensis*, *N. transvalensis*, *Actinomadura peletieri*, *A. madurae*, *Streptomyces somaliensis*, *S. griseus* and *Nocardiopsis dassonvillei*. The remaining 11 isolates were identified at the genus level including *Nocardia* (five isolates), and *Actinomadura* and *Streptomyces* (each 3 isolates). Table **1** shows the numbers, diversity and prevalence of the actinomycete populations in soil samples from these 25 locations. A total of 34 (35.4%) *Nocardia* isolates, representing four species, were identified. Of which, *N. asteroides* surpassed the other three species of *Nocardia*, accounting for 44.1% of all *Nocardia* species, and ranked third in prevalence (15.6% of all isolates). All *Nocardia* isolates exhibited partial acid-fastness, varying from weak to strong. Colony morphology of *Nocardia* spp. were glabrous, wrinkled or granular with color of orange, yellow to white. Regarding *N. transvalensis*, both of the isolates tested decomposed hypoxanthine, tyrosine, and urea and were negative for casein decomposition.

Table 1. Prevalence and Diversity of Acinomycetes Isolated from 300 Soil Samples in Qazvin City

Actinomycetes Species Isolated	Number	Percent
Actinomadura madurae	19	19.8
Streptomyces somaliensis	19	19.8
Nocardia asteroides	15	15.6
N. otitidiscaviarum	9	9.4
N. brasiliensis	7	7.3
Actinomadura pelletieri	5	5.2
Streptomyces griseus	5	5.2
Nocardia spp.	5	5.2
Actinomadura spp.	3	3.1
Streptomyces spp.	3	3.1
Nocardia transvalensis	3	3.1
Nocardiopsis dassonvillei	3	3.1
Total	96	100

The *Streptomyces* colonies were slow growing, aerobic, folded, glabrous or chalky, with earthy odour and aerial mycelia of different colors. All streptomycete isolates were acid-fast negative and Gram-stain positive. The degradation of the substrates casein and tyrosine by *S. somaliensis* and *S. griseus* was positive but for xanthine was variable. Microscopically, it was observed that the morphology of the spore chains varied depending on the species, which was straight or flexuous, hook or coil form.

N. brasiliensis, *Nocardiopsis dassonvillei*, *S. griseus* and *S. somaliensis* were the species capable of liquefying gelatin. *N. transvalensis* and *Nocardiopsis dassonvillei* were the only species could breakdown starch. The percentage of species may be presented by overall rate (positive number/300 soil samples).

DISCUSSION

In this study, aerobic actinomycetes present in soil were isolated frequently from soil of different parts of Qazvin province. Actinomycetes may cause various infections such as nocardiosis and actinomycetoma, which deserve special attention but delay in diagnosis result in deterioration of the patient's condition and mortality rate, especially in cases of metastatic nocardiosis.

In the Finnish Health Authority guidelines, existence of spore-forming actinobacteria in the environments has been considered as a risk to human health [1]. The literature is crowded with case reports on actinomycotic infections especially mycetoma. Several reports from various geographical locations of Iran have shown that actinomycotic mycetoma is a sporadic disease, frequent etiological agents of which are *N. asteroids* and *Actinomadura madura* [7-11]. In this study, out of 300 soil samples, 96 actinomycete isolates (32%) yielded, which *A. madurae* and *S. somaliensis* were of the predominant isolates (Table **1**). Whereas in a study carried out in Rome, out of 200 soil samples examined, only 12 actinomycete species (6%) including seven isolates of *N. asteroides*, four of *A. madurae* and one of *Nocardiopsis dassonvillei* were isolated [12]. According to a study carried out on 64 cases of actinomycetomas over 33 years in Iran, *Actinomadura madurae* has been the cause of 10.2% of the cases [7]. Furthermore, Denguezli *et al*. has reported that of 10 actinomycetoma cases from Tanzania, nine cases were caused by *A. madurae* [13].

In this study, 40.6% of soil samples in Qazvin, contained different species of *Nocardia*, which is much higher than what reported in Patiala, India, that is 8% [14]. *Nocardiae* are ubiquitous in the environment and well known as saprophytic soil inhabitants. Identification of *Nocardiae* to the species level appears to be of significant benefit to the patients' management and defining the geographic distribution of these organisms. Members of the genus *Nocardia* are most commonly associated with a diverse array of opportunistic infections in both immunocompromised and immunocompetent hosts [15]. In a study carried out during 1972 to 2005 in Iran, most of (84.5%) all mycetoma cases were actinomycetoma [7], which is in concordance with the results of Dieng *et al*. and Denguezli *et al*. [13,16]. From the available data, mycetomas were abundant among farmers in rural areas of Iran and the disease mainly has been seen in foot, which shows that the causative agents mostly implanted from soil or plants traumatically [7, 8, 10]. Similar results have been found in Argentina [17] and Tanzania [13].

Most of the nocardiosis cases are caused by *N. asteroides* [15] and in the current study, this species surpassed the other *Nocardia* species and had allowed it to become the most common *Nocardia* species isolated from soil. These results are comparable with those of the Kuwaiti and Italian studies [12, 18]. *Nocardiae* are usually acquired through inhalation and infrequently by direct skin inoculation [15]. A study from South India showed that contact with soil and sands contaminated with *N. asteroides* has been the reason for 1.4% of corneal lesions cases [19]. Nocardiosis produced by *Nocardia* spp. present in soil were unusual in the past, but

they are in increase in recent years with the increase in the number of immune deficiency patients [20]. Curry (1980] stated that 38 percent of people inflicted with pulmonary nocardiosis lacked any background [21]. Different forms of Nocardiosis in various immunocompromised patients have been recognized in Iran [personal communication]. Over the past decades in Iran, the frequency of pulmonary nocardiosis infections caused by *N. asteroides* has increased [22-25]. These patients typically had predisposing factors such as pemphigus vulgaris, kidney transplantation, chronic lymphocytic leukemia and Wagner vasculitis. According to Munoz *et al*. [26], *N. asteroides* and *N. brasiliensis* have been the most common agents of nocardiosis in HIV-positive patients.

In this study, *N. otitidiscaviarum* was the second most prevalent *Nocardia* spp. and was accounted for 26.7% of all *Nocardia* species, but based on the literature, this species has infrequently been recovered from soil throughout the world. It has also been postulated that *N. otitidiscaviarum* is less pathogenic than other species of *Nocardia* [27] and represented only between 1.3 and 4.8% of clinical *Nocardia* isolates in the studies from Europe, Japan and the USA [28]. Based on a study on 264 actinomycetoma patients from west of Bengal, *N. otitidiscaviarum* has had a lower incidence of etiological agents [29].

In our study, *Nocardia brasiliensis* constituted 20.6% of all *Nocardia* species. This *Nocardia* species produces infection most frequently in immunocompromised patients, but it can cause cutaneous and disseminated infection in immunocompetent patients too [30]. A review of actinomycetomas cases from 1972 to 2005 in Iran demonstrated that *N. brasiliensis* has been the cause of 86.6% of the cases, which have mostly been associated with tropical environments [7]. In the United States also, *N. brasiliensis* is the second most common isolate of *Nocardia* [6].

In the current study, *N. transvalensis* and *Nocardiopsis dassonvillei* were the uncommon (each 3.1%) actinomycete species isolated. It has been stated that the specific natural habitat of *N. transvalensis* has not yet been identified [6]. *Nocardiopsis* species have been observed in indoor environments and shown to produce antimicrobial and bioactive agents. *Nocardiopsis dassonvillei* isolated from lung biopsies of farmers suffering from alveolitis [3].

This is the first report on isolation and characterization of medically important aerobic actinomycetes in soil of Iran. This study can possibly reveal actinomycetes agents that engaged in actinomycotic diseases in Iran. Familiarity with the epidemiology of unusual *Nocardia* species may increase early identification in cases of nocardial infection. Moreover, it is obvious that efforts for the isolation, characterization and study on actinomycetes from different ecological niches can be a milestone for the discovery of novel species that may yield potential human and veterinary pathogens.

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