Molecular analysis and species diversity of *Nocardia* in the hospital environment in a developing country, a potential health hazard

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**Abstract**

**Purpose.** Despite hundreds of reports on the isolation of *Nocardia* from clinical samples, the presence and diversity of *Nocardia* species that are capable of survival in a harsh and adverse condition, such as a hospital environment, have not been comprehensively studied. The aim of this study was to assess *Nocardia* species diversity in a hospital environment to provide a better insight into their potential threat as a reservoir for the development of nosocomial infections.

**Methodology.** A total of 90 samples of hospital water, dust and soil, collected from 30 hospitals, were analysed for the presence of *Nocardia* using standard protocols for isolation and characterization of the isolates. Conventional tests were used for preliminary identification, and PCR amplification of the 596 bp amplicon of the 16S rRNA and sequence analysis of 16S rRNA were performed for genus and species identification.

**Results.** A total of 25 *Nocardia* isolates (27.7 %) from 10 species were recovered from 90 samples. The three most prevalent species were *N. cyriacigeorgica*, 24 %, *N. asteroides*, 16 % and *N. kroppenstedtii*, 12 %, followed by *N. salmonicida*-like, 8 % and single isolates of *N. otitidiscaviarum*, *N. flavoroezae*-like, *N. neocaledoniensis*-like and *N. sungurluensis*-like. Thirteen out of twenty five isolates showed characteristics of six novel species.

**Conclusion.** Our study showed that the hospital environment is a potential reservoir of a diverse range of *Nocardia* species, due to the remarkable survival capability of these bacteria in an adverse hospital environment, which carries a threat to the health of patients.

**INTRODUCTION**

*Nocardia* was first isolated and described in 1888 from cases of bovine farcy in cattle by Edmond Nocard and, a year later, Trevisan coined the name *Nocardia* for the genus that consisted then of five species [1, 2]. Two years later, Eppinger reported the first case of human infection by fatal disease called pseudotuberculosis [3], based on brain pathology the organism was named *Nocardia asteroides*, and assigned as the ‘type species’ for the genus *Nocardia* [4]. Ever since there has been a plethora of case reports of diseases caused by *Nocardia* in humans and in a large number of animals [5, 6]. The annual incidence of nocardiosis in North America between 1970 and 2005 has been estimated at 0.001–1.84 %. The lowest and highest rates were recorded in Canada and Mexico, respectively. The corresponding rates in South America have generally been higher: 1.8 % (Colombia) to 2 % (Chile), while those in Europe and Asia are almost the same as for North America: 0 % (Iceland) to 1.9 % (Romania) and 0.001 (United Arab Emirates) to 1.9 % (China). The highest incidence of nocardiosis has been found in Africa: 1.8 (Congo) to 4.1 % (Nigeria) [7].

In Iran, the corresponding rate of nocardiosis has been estimated to be 1.88 % [7]. Further to this report, our literature review revealed that in that country there have been very few studies dealing with the isolation of *Nocardia* from clinical samples. The rates of isolation of *Nocardia* in these studies have been estimated to be 3.1 to 28.1 % [8–12].

*Nocardia* causes a variety of acute and chronic infections in humans and animals that vary from self-limiting inapparent
infection to chronic disseminated diseases that are difficult to treat [13, 14]. The most common form of disease is pulmonary infection, accounting for 75 % of cases, followed by central nervous system nocardiosis which develops in 15–40 % of the patients with pulmonary infection. This is followed by cutaneous infection due to traumatic inoculation of the organism in immunocompetent individuals [13, 15, 16]. Almost all nocardiosis cases occur in an immunocompromised host or in patients with underlying pulmonary disease with no distinguishable characteristics that are often misdiagnosed as a viral, mycoplasmal, fungal or tuberculosis infection or various forms of cancer [5, 17, 18].

There is no absolute evidence for person-to-person transmission of Nocardia; however, the hardy nature and ubiquitousness of Nocardia in the environment, mainly in soil, air, dust or contaminated water [14, 19, 20], allow it to survive in the environment of healthcare facilities and thereby to invade susceptible hosts [21–23].

Despite the abundance of reports in the scientific literature regarding the isolation of Nocardia from both clinical samples and hospitalized patients [15–18, 22], no study has yet investigated the extent of the presence and diversity of Nocardia species capable of survival in the harsh and adverse conditions of the hospital environment. The aim of this study was to assess the frequency and diversity of Nocardia species capable of survival in the hospital environment, by applying molecular and conventional microbiologic methods in order to provide a better insight into their likely role as a reservoir for the transmission and development of nosocomial infections.

METHODS

Sampling

A total of 90 environmental samples were collected from dust, soil and water resources of 30 hospitals in the second most developed province of Iran, Isfahan, between October 2013 and November 2014. Sampling and pretreatment were carried out according to standard methods [24]. In brief, soil samples (1 g) were stirred for 30 min in 100 ml sterile Ringer’s solution (5 % v/v). For the dust samples, 2–3 g dust was collected from the floors and window panes of patients’ rooms with a sterile swab or brush, suspended in 100 ml distilled water for 60 min, and allowed to stand at room temperature for additional 10 min. Tenfold dilutions of the homogenized suspensions were prepared and 200 µl of each of the pretreated 10^{-2}, 10^{-3} and 10^{-4} dilutions were inoculated into Sauton’s media supplemented with antifungal and antibacterial antibiotics [kanamycin, nystatin and nalidixic acid (each at 50 µg ml^{-1})] and incubated for 3 weeks at 25, 32 and 37 °C [25].

The water samples were collected in volumes of 1 l, decontaminated with cetypyridinium chloride 0.005 % for 15 min and vacuum filtered using cellulose nitrate filters (0.45 µm, Sartorius AG). The filters were rinsed and macerated in tubes containing 15 ml distilled water. Aliquots of approximately 100 µl of dissolved samples were transferred into Sauton’s medium, and incubated as described for the soil samples.

Conventional bacterial identification

The environmental isolates were identified primarily as Nocardia by conventional phenotypic tests including partial acid-fast staining, growth at 25, 32 and 37 °C, pigment production and standard biochemical assays, including resistance to lysozyme, hydrolysis of tyrosine, xanthine and hypoxanthine tests [22, 26]. The identification was further pursued by molecular testing as follows.

Molecular identification

Chromosomal DNA was extracted using a simple boiling method. In brief, several colonies of bacteria were added to 200 ml TE buffer, boiled for 30 min and centrifuged at 11 180 g for 10 min. The supernatant was transferred to another sterile microtube and centrifuged at 20 000 g for 10 min. Precipitated DNA was resuspended in 50 µl Milli-Q water and stored at −20 °C.

The isolates identified phenotypically as Nocardia were further analysed to the genus and species levels using a panel of molecular tests that included a genus-specific PCR based on a 596 bp region of the 16S rRNA as recommended by Laurent [27], followed by the amplification and direct analysis of almost complete 16S rRNA sequencing for species identification as described by Roth [28]. Sequencing was performed by the Bioneer company (South Korea), and the sequence data received were aligned manually with existing sequences of Nocardia retrieved from the GenBank database and analysed using the Blast program in GenBank and the jPhydit program [29].

RESULTS

In this study, a total of 25 (27.7 %) Nocardia isolates were recovered from 90 water and soil samples collected from the hospitals. The positive samples came from the 19 of the 30 hospitals that participated in the study. No Nocardia isolates were detected in any of the analysed samples taken from the other 11 hospitals. Of the total isolates, 19 strains were recovered from soil and dust samples and 6 strains were recovered from water samples.

The recorded temperature of the soil samples was in the range of 11 to 18 °C and the pH of the samples was between 7.5 and 9.0. The corresponding figures for water samples were 14–29 °C and 7.5–8.5, respectively. The total dissolved solids for the water samples in indoor areas ranged between 365 and 412 mg l^{-1}.

The details of water, dust and soil samples and the isolates, key properties are shown in Table 1.

Based on morphological, culture and biochemical properties, including resistance to lysozyme, pigmentation and decomposition of tyrosine, xanthine and also the PCR-based genus-specific marker, i.e. the presence of a 596 bp
Table 1. Samples profile phenotypic and molecular features of *Nocardia* isolates from Iranian hospital environments

| Isolate | Hospital  | Department (hospital location) | Hospital designation and location: hospitals 1, 2, 3, 4, 6, 8, 9, 10, 11, 14, 15, 16, 17 and 18 are located in Isfahan city; 5, Zarrin Shahre, 40 km south of Isfahan; 7, Shahin Shahre, 24 km north of Isfahan city; 12, Ferydoon Shahre, 150 km west of Isfahan city; 13, Fulad Shahre, 25 km south-west of Isfahan; 19, Flavarjan city, 24 km south-west of Isfahan. | Sample profile | Phenotypic features | 16S rRNA analysis |
|---------|-----------|---------------------------------|------------------|-----------------|-----------------|
|         | Hospital* | Department                       | Source | pH | Temperature | Lysozyme resistance | Pigment | Decomposition of tyrosine | Decomposition of xanthine | Decomposition of hypoxanthine | Optimum temperature (°C) | Similarity (%) | Base pair differences | Identification |
| 1       | NR2       | 1 Male surgery                     | Dust   | 7.7 | 15          | –                  | + Pink | –                         | –                         | –                         | 35               | 100            | 0.847            | *N. asteroides* |
| 2       | NR3       | 2 Emergency                        | Dust   | 7.8 | 14          | –                  | + Yellow | –                         | –                         | –                         | 35               | 100            | 0.816            | *N. kroppenstedti* |
| 3       | NR4       | 3 Outdoor area                     | Soil   | 8.2 | 18          | –                  | + White | –                         | +                         | +                         | 35               | 100            | 0.824            | *N. otitidiscaviarum* |
| 4       | NR5       | 4 Outpatient clinic                 | Water  | 7.5 | 29          | 412                | + Pink | +                         | –                         | –                         | 25               | 99.5           | 6/1390            | *N. flavissima-like* |
| 5       | NR6       | 3 Oncology                         | Dust   | 8.5 | 11          | –                  | + White | –                         | –                         | –                         | 35               | 100            | 0.807            | *N. cyriacigeorgica* |
| 6       | NR7       | 5 Outdoor area                     | Soil   | 9   | 18          | –                  | + Yellow | –                         | –                         | –                         | 30               | 99.5           | 6/812             | *N. coublieae-like* |
| 7       | NR9       | 6 Orthopaedics                      | Dust   | 7.7 | 15          | –                  | + Pink | –                         | –                         | –                         | 35               | 100            | 0.847            | *N. asteroides* |
| 8       | NR11      | 7 Outdoor area                     | Soil   | 7.8 | 12          | –                  | + Pink | –                         | –                         | –                         | 35               | 99.23          | 3/778             | *N. asahinae-like* |
| 9       | NR13      | 7 Emergency                        | Dust   | 7.8 | 12          | –                  | + Pink | –                         | –                         | –                         | 35               | 99.71          | 4/1363            | *N. asahinae-like* |
| 10      | NR14      | 6 Outdoor area                     | Soil   | 7.7 | 15          | –                  | + Pink | –                         | –                         | –                         | 35               | 100            | 0.847            | *N. asteroides* |
| 11      | NR16      | 8 Neurosurgery                     | Water  | 7.5 | 29          | 486                | + White | +                         | –                         | –                         | 25               | 99.5           | 6/1390            | *N. flavissima-like* |
| 12      | NR17      | 9 Infectious diseases              | Water  | 7.6 | 12          | –                  | + White | –                         | –                         | –                         | 35               | 100            | 0.981            | *N. cyriacigeorgica* |
| 13      | NR18      | 9 Ophthalmology                    | Water  | 7.2 | 21          | 365                | + White | –                         | –                         | –                         | 35               | 100            | 0.861            | *N. cyriacigeorgica* |
| 14      | NR19      | 10 Outdoor area                    | Soil   | 7.8 | 14          | –                  | + Yellow | –                         | –                         | –                         | 35               | 100            | 0.816            | *N. kroppenstedti* |
| 15      | NR20      | 11 Outdoor area                    | Soil   | 7.7 | 15          | –                  | + Pink | –                         | –                         | –                         | 35               | 100            | 0.847            | *N. asteroides* |
| 16      | NR21      | 12 Outdoor area                    | Soil   | 8.6 | 11          | –                  | + Yellow | –                         | +                         | 30                      | 98.92           | 8/740          | *N. cshihjams-like* |
| 17      | NR23      | 13 Male surgery                    | Dust   | 8.2 | 12          | –                  | + Pink | –                         | +                         | 30                      | 99.25           | 7/939          | *N. sungurovii-like* |
| 18      | NR25      | 8 Male surgery                     | Water  | 7.5 | 20          | 365                | + White | –                         | –                         | –                         | 35               | 100            | 0.807            | *N. cyriacigeorgica* |
| 19      | NR26      | 14 Maternity                       | Dust   | 7.6 | 13          | –                  | + White | –                         | –                         | –                         | 35               | 100            | 0.910            | *N. cyriacigeorgica* |
| 20      | NR28      | 2 Outdoor area                     | Soil   | 7.8 | 12          | –                  | + White | –                         | –                         | –                         | 35               | 100            | 0.916            | *N. cyriacigeorgica* |
| 21      | NR30      | 15 Outdoor area                    | Soil   | 7.8 | 14          | –                  | + Yellow | –                         | –                         | –                         | 35               | 100            | 0.847            | *N. kroppenstedti* |
| 22      | NR32      | 16 Internal medicine               | Water  | 7.5 | 29          | 514                | + Pink | +                         | –                         | –                         | 25               | 99.8           | 2/995             | *N. flavissima-like* |
| 23      | NR33      | 17 Outdoor area                    | Soil   | 8.3 | 16          | –                  | + White | –                         | –                         | –                         | 35               | 99.25          | 6/800             | *N. flavissima-like* |
| 24      | NR34      | 18 Maternity                       | Dust   | 9   | 18          | –                  | + White | –                         | –                         | –                         | 30               | 99.5           | 6/880             | *N. coublieae-like* |
| 25      | NR30      | 19 Paediatrics                     | Dust   | 9   | 18          | –                  | + White | –                         | –                         | –                         | 30               | 98.5           | 15/1390           | *N. coublieae-like* |

†Hospital designation and location: hospitals 1, 2, 3, 4, 6, 8, 9, 10, 11, 14, 15, 16, 17 and 18 are located in Isfahan city; 5, Zarrin Shahre, 40 km south of Isfahan; 7, Shahin Shahre, 24 km north of Isfahan city; 12, Ferydoon Shahre, 150 km west of Isfahan city; 13, Fulad Shahre, 25 km south-west of Isfahan; 19, Flavarjan city, 24 km south-west of Isfahan.

‡TDS, Total Dissolved Solids.

§Similarity: % similarity to the nearest validated species.

¶Base pair difference: the number of nucleotide differences between the isolate and the nearest validated species.
amplicon of 16S rRNA, all 25 isolates were identified as Nocardia (Table 1). The 16S rRNA gene sequencing of the isolates revealed that all isolates had nucleotide signatures of mycobacteria at positions 70-98 (U-A), 139-224 (G-C), 843 (C), 1008-1021 (C-G), 1189 (C), 1244-129 (C-G) and 1308-1329 (C-G) [30, 31].

The three most prevalent Nocardia species isolated from hospital environments were Nocardia cyriacigeorgica, six isolates (24 %); N. asteroides, four isolates (16 %) and Nocardia kroppenstedtii, three isolates (12 %). These were followed by three unknown species that included three isolates, each (12 %) of the strains closely related to Nocardia coublieae, and Nocardia fluminea and two isolates (8 %) closely related to Nocardia salmonicida, respectively. The remaining four isolates belonged to one established species, Nocardia otitidiscaviarum, and three unknown Nocardia species closely related to N. flavoroeza, N caishijienis and N sungurluensis (Table 1).

The almost complete 16S rRNA gene sequences obtained for the accurate identification of species of Iranian isolates showed that isolates NR25, NR17, NR18, NR6, NR26 and NR28 had 100 % similarity with the type strain of Nocardia otitidiscaviarum, DSM 44484; isolates NR20, NR14, NR9 and NR2 showed 100 % similarity with N. asteroides type strain DSM 43757; isolate NR4 showed 100 % similarity with N. otitidiscaviarum type strain DSM 43242; and isolates NR3, NR30 and NR19 showed 100 % similarity with N. kroppenstedtii type strain DSM 45810.

Isolates NR7, NR34 and NR50 had 98.5–99.5 % similarity (corresponding to 6–15 bp nucleotide differences) with N. coublieae type strain DQ235688; isolates NR11 and NR13 had 99.71 % similarity (corresponding to 4 bp nucleotide differences) with N. salmonicida type strain JCM 282630; isolates NR32, NR16 and NR5 had 99.5–99.8 % similarity (corresponding to 2–6 bp nucleotide differences) with N. fluminea type strain JCM 11440; isolate NR33 showed 99.25 % similarity (corresponding to 6 bp nucleotide differences) with N. flavoroeza type strain JCM 3322; the isolate NR21 showed 98.95 % similarity with N. caishijienis type strain JCM 11508 (corresponding to 8 bp nucleotide differences); and isolate NR23 showed 99.25 % similarity (corresponding to 7 bp nucleotide differences) with N. sungurluensis type strain CR3272. The magnitude of nucleotide differences between our isolates and the established Nocardia species make them candidates for further investigation as novel species. All these isolates had a unique signature nucleotide sequence at the hyper-variable region, positions 137 to 193 (E. coli numbering system) (Table 2).

The relationship between our isolates and the established standard species of Nocardia was supported by a high bootstrap value in our phylogenetic tree based on the 16S rRNA gene, while the corresponding value for isolates that showed substantial nucleotide differences with known Nocardia species, and named candidates as novel species requiring further investigation, was found to be rather low (Fig. 1).

DISCUSSION

The genus Nocardia is part of the family Nocardiaceae that also includes the genera Gordonia, Rhodococcus and Skermania, forming the distinct actinomyocyte phylogenetic line that contains mycolic acid and is morphologically characterized by the formation of hyphae. In the last decade over half of the 111 species within the genus Nocardia have been described. This is in addition to the eight species (N. asteroides, Nocardia farcinica, N. cyriacigeorgica, Nocardia nova, Nocardia brasiliensis, N. otitidiscaviarum, Nocardia pseudobasilensis and Nocardia transvalensis) that are historically associated with Nocardia infections in humans [13, 15–18, 22].

Infection caused by Nocardia species received greater clinical attention in recent years [18, 22, 23]. The isolation and characterization of over 17 novel species from clinical samples in the last decade is documented evidence of this claim [32].

Apart from spore-forming bacteria such as Bacillus and Clostridium, which are highly resistant when disseminated as spores, acid-fast and partially acid-fast bacteria such as mycobacteria and Nocardia have protective cell walls containing thick peptidoglycan and mycolic acids that render them relatively resistant to thermal killing and chemical disinfection [33–35]. This important characteristic confers upon Nocardia its ability to survive well in harsh environments such as those found in hospitals. This species thus carries a threat to patient care and, in particular, to immunocompromised patients. Certain patient risk factors, including advanced age, underlying disease and severity of illness, and sometimes immune status, cannot be modified and directly contribute to the patient’s risk of acquiring a nocardial infection [18, 36].

Microbial assessment of the hospital environment, particularly in developing countries, does not include examination for the presence of Nocardia. On the other hand, because the routine procedures used in laboratories for isolation and identification of microbial agents are not able to identify the Nocardia species, no attempts have been made to control Nocardia in the hospital environment [37]. However, several studies have revealed that contamination of hospital equipment and medical supplies, traced to the persistence of Nocardia in soil, dust, water and their resistance to commonly used disinfectants, was responsible for outbreaks of infections associated with surgical implants, health care-associated septicaemia and lung disease following bronchoscopy [38, 39].

The aims of the current study were (1) to determine the extent of the presence and diversity of Nocardia in the hospital environment, through the optimization of isolation methods; and (2) the accurate identification of Nocardia species recovered from environmental sources in hospitals using a combination of phenotypic and molecular methods.
Table 2. Alignment of selected stretches of signature nucleotide regions (positions 137–193; E. coli numbering) in the 16S rRNA of strains of *Nocardia* isolated from Iranian hospital environments with those of reference type species

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*Indicates that the base pair is identical to that of the type strain of *N. asteroides*.

*16S rRNA positions according to *E. coli* numbering system.
Fig. 1. 16S rRNA sequence-based phylogenetic tree for Iranian hospital environment Nocardia isolates and nearest validated species of Nocardia using the neighbour-joining method. The figures at each node represent bootstrapping values. The tree was rooted with Gordonae terrae.
In the current study, we isolated and characterized 25 Nocardia species from 90 samples of water, soil and dust collected from 30 hospitals. The isolation rate was 27.7%, which is within the rate reported for the environmental isolation of Nocardia by studies from Kazakhstan and the UK [40, 41]. However, this rate is high compared to findings reported from Thailand [42], and low compared to those reported from Kuwait and Brazil [43, 44]. The Nocardia species recovered from hospital environments in our study included four opportunistic pathogens and six potentially novel species with unknown human pathogenicity.

In our study, N. cyriacigeorgica was the most commonly encountered Nocardia, representing 24% of the isolates. This organism is a human opportunistic pathogen that was first isolated and characterized, in 2001, from the bronchial secretions of a patient with chronic bronchitis [45]. N. kroppenstedtii ranked second, representing 16% of the isolates. This is the first historically identified Nocardia species and has been the most commonly reported isolate from clinical samples world wide [18]. N. kroppenstedtii ranked third, representing 12% of the isolates. This species is a human opportunistic pathogen that was first isolated and characterized in 2014, from a lung transplant patient with a pulmonary infection [46].

In our study, only a single isolate of N. otitidiscarvum was recovered from the hospitals. This organism is a human opportunistic pathogen that was first isolated and characterized in 1924 from clinical samples [47]. It is ubiquitous in water and soil and has frequently been recovered from both clinical and environmental samples [48, 49]. Thirteen isolates showed molecular and phenotypic characteristics different from all previously established Nocardia. These isolates comprised six potentially novel Nocardia species, since they had 16S rRNA nucleotide sequences corresponding to closely related Nocardia species. The characterization of these unknown Nocardia species remains to be completed, using a thorough phenotypic and molecular analysis including cell wall composition analysis, gyrB, secA1, hsp65, and rpoB and DNA–DNA relatedness [50–52].

**Conclusion**

In conclusion, the results of current study show that hospital soil and water resources, including potting soil, garden soil and dust on window frames and equipment, and both potable and non-potable water, may carry a wide range of Nocardia. These resources thus represent a reservoir and potential source for transmission of these opportunistic bacteria to susceptible persons, and in particular to those suffering from life-threatening diseases such as cancer or organ transplantation. Nosocomial infection control strategies should focus on the proper cleansing of the environment to ensure the removal of lesser-known bacteria such as Nocardia from hitherto underestimated reservoirs, including dust in the critical wards or water used directly or indirectly for patient care.

**Funding information**

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**

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