Molecular identification and susceptibility pattern of clinical Nocardia species: Emergence of Nocardia crassostreae as an agent of invasive nocardiosis

Saad J Taj-Aldeen PhD D(ABMM)1, Anand Deshmukh MD D(ABMM)1, Sanjay Doiphode MD FRCPath1, Atqah Abdul Wahab MD FCCP2, Mona Allangawi MD3, Ahmed AlMuzrkchi MD4, Corné H Klaassen PhD 5, Jacques F Meis MD PhD5

BACKGROUND: Nocardia species are rare, opportunistic organisms that cause disease in both immunocompetent and immunocompromised individuals.

OBJECTIVE: To investigate the clinical presentations of various Nocardia infections based on the 16S ribosomal RNA gene of the isolate, as well as related risk factors and susceptibility patterns to antimicrobial agents

METHODS: Thirteen patients with a diagnosis of nocardiosis were included in the present study. Seven Nocardia species were identified by 16S ribosomal RNA. Susceptibility testing was performed using six antimicrobial agents.

RESULTS: Five patients were immunocompromised, and eight were immunocompetent with predisposing factors including cystic fibrosis, tuberculosis and ophthalmic infections. Nocardia caused pulmonary infections in eight patients (61.5%), invasive systemic infections in three patients (23%) and local (ophthalmic) infections in two patients (15.4%). In the patients with pulmonary disease, nocardiosis was caused by six species (Nocardia cyriacigeorgica, Nocardia otitidiscaviarum, Nocardia farcinica, Nocardia carnea, Nocardia testacea and Nocardia asiatica). The seventh species identified in the present study was Nocardia crassostreae.

DISCUSSION: N crassostreae is a multidrug-resistant organism that was reported to be an emerging human pathogen causing invasive nocardiosis in a patient with non-Hodgkin’s lymphoma. N farcinica was isolated from blood in a patient with breast cancer. None of the Nocardia isolates were resistant to linezolid. One N otidiscaviarum isolate was a multidrug-resistant organism. All patients in the present study were treated with the appropriate antibiotics and their condition resolved without further sequelae.

CONCLUSIONS: The present study is the first report on N crassostreae as a human pathogen. The detection of multidrug-resistant species necessitates molecular identification and susceptibility testing, and should be performed for all Nocardia infections. Nocardiosis manifests various clinical features depending on the Nocardia species and underlying conditions.

Key Words: Antibiotic susceptibility; Clinical cases; Molecular identification; Nocardia; Nocardia crassostreae


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Nocardioid are ubiquitous organisms distributed worldwide in the environment as saprophytic components of fresh and salt water, soil, dust, decaying vegetation and decaying fecal deposits from animals (1). Nocardioid are rare opportunistic organisms that cause diseases in immunocompetent and immunocompromised individuals, and are reported in all ages and ethnic groups. Immunocompetent patients usually develop localized cutaneous lesions, such as cellulitis, abscesses or sporotrichoid forms (2), and endogenous endophthalmitis (3). However, most cases are reported in immunocompromised patients, and manifest as deep infections or disseminated diseases (4-6). Risk factors for infection with Nocardioid include solid organ transplant (5,7-9) or bone marrow transplant recipients (10), patients with hematomatologic diseases (11,12) and HIV infection (13-16). Pulmonary nocardiosis can be a cause of disease in populations with risk factors such as immunosuppression, malignancies and severe lung disease (17), patients with cystic fibrosis (18), and in patients with chronic obstructive pulmonary disease and bronchiectasis (19,20). Lung infections are frequent and, in many cases, disease can spread to the central nervous system, including the brain (4,21), with poor prognosis in some cases, irrespective of antimicrobial therapy (22).

Identification of clinical isolates beyond the genus level is important because Nocardioid species differ in clinical spectrum and their susceptibility to antibiotics. The classic laboratory methods of identification, including direct examination and culture, are insufficient; therefore, sequence analysis of the 16S rDNA gene is mainly used for the identification of Nocardioid to species level (23,24).

Trimethoprim-sulfamethoxazole has traditionally been the agent of choice for the treatment of nocardiosis, with alternative drugs including amikacin and imipenem (1,25). Resistance and therapeutic failure may occur, which necessitates a search for alternative agents. The aim of the present study was to investigate the clinical presentations of various Nocardioid infections based on the 16S rDNA gene of the isolate, related risk factors and susceptibility patterns to various antimicrobial agents.

METHODS

Patients
The present laboratory-based study was approved by the Scientific Council and Ethics Committee of Hamad Medical Research Center, Doha, Qatar (proposal number 10174/10).

Thirty patients of different nationalities with various clinical symptoms and risk factors admitted to Hamad Hospital, Doha, Qatar, were diagnosed with nocardial infections by the main Microbiology Laboratory of Hamad Medical Corporation from January 2006 to June 2010. Patients’ clinical records, demographic data and treatment outcomes were included in the present study. Information regarding sex, age, underlying conditions including history of immunosuppressant drug use, tuberculosis and malignancy were analyzed (Table 1). Disseminated nocardiosis was considered for infection of two organs or more, such as lungs, lymph nodes, brain or blood. The respiratory samples included sputum, endotracheal aspiration and bronchoalveolar lavage. A diagnosis of pulmonary nocardiosis required at least one positive culture from respiratory samples, and the presence of clinical symptoms and an abnormal chest radiograph.

Isolation and identification of Nocardioid species
A total of 13 clinical specimens positive for Nocardioid species were recorded over a four-year period. Nocardioid species were isolated and identified according to standard laboratory procedures. Identification of Nocardioid species was based on Gram-positive branching, beaded and filamentous bacilli, and positive modified acid-fast stain results. The clinical specimens were generally cultured on chocolate agar and blood agar media, and incubated in both aerobic and anaerobic conditions at 37°C. Characteristic dry, chalk-like Nocardioid colonies appeared on aerobic cultures after three to seven days of incubation, depending on the species. Blood cultures were performed using the Bactec automated culturing system (BD Diagnostic Systems, USA).

Molecular analysis
The identities of the clinical isolates were further confirmed by 16S rDNA gene analysis (26). The DNA was isolated from freshly frozen colonies using a MagNA Pure LC instrument in combination with MagNA Pure LC DNA isolation kit III according to the instructions of the manufacturer (Roche Diagnostics, The Netherlands). An approximately 500 bp fragment from the 5’ end of the 16S rRNA gene was amplified using polymerase chain reaction containing 1 U of FastStart Taq DNA polymerase (Roche Diagnostics), 0.2 mM dNTPs, 1.5 mM MgCl2, and 0.5 µM of both amplification primers (forward: 5’-CTT AAT AAC ACA TGC AAG TCG ARC G-3’; reverse: 5’-CTT ATT ACC GCC GCT GCC TCT-3’) in 1x polymerase chain reaction reaction buffer. Cycling conditions were as follows: 30 s at 94°C, 30 s at 56°C and 1 min at 72°C repeated 30 times, preceded by a 10 min activation step at 94°C and followed by an additional 10 min elongation step at 72°C. The amplified product was purified using SPIRI chemistry (AMPure, Beckman Coulter, The Netherlands) and subjected to DNA sequence analysis with the reverse amplification primer using the DyeNamic ET dye terminator kit (GE Healthcare, Belgium) as recommended. Sequence reaction products were purified using SPIRI chemistry (CleanSeq Beckman Coulter) and analyzed on a MegaBACE 500 automated DNA analysis platform (GE Healthcare) using standard electrophoretic conditions. The obtained sequences were verified and manually corrected when necessary using MegaBACE Sequence Analyzer v3.0 (GE Healthcare). Sequences were then compared with the public DNA databases using the BLAST interface (www.ncbi.nlm.nih.gov/BLAST).

Susceptibility testing
Antimicrobial susceptibility testing was performed using Etest (AB biodisk, Sweden). A suspension of the microorganism, with turbidity equivalent to 1.2 McFarland standard, was inoculated (150 µL/plate) by confluent swabbing on Mueller-Hinton agar plates. A maximum of two Etest strips were applied to each plate. Etest plates were incubated at 35°C and results were recorded after 48 h (or after 72 h if growth was insufficient after 48 h). The following antimicrobial agents were tested (concentration ranges): amikacin (0.016 µg/mL to 256 µg/mL), moxifloxacin (0.002 µg/mL to 32 µg/mL), cefotaxime (0.016 µg/mL to 256 µg/mL), ciprofloxacin (0.002 µg/mL to 32 µg/mL), linezolid (0.016 µg/mL to 256 µg/mL) and imipenem (0.002 µg/mL to 32 µg/mL). Agents were determined by Etest for 13 Nocardioid isolates from clinical specimens. Minimum inhibitory concentrations were determined according to manufacturer’s guidelines. Results were interpreted as susceptible, intermediate or resistant according the breakpoints recommended by the Clinical and Laboratory Standards Institute for Nocardioid and other aerobic actinomycetes (27).

Beta-lactamase activity: All Nocardioid clinical isolates were tested for beta-lactamase activity using the nitrocefin disk test.

RESULTS

Microbiological investigation
A total of 13 Nocardioid isolates were identified from 13 patients during the period January 2006 to June 2010. Molecular identification yielded Nocardioid cyriacigeorgica (n=3; Genbank accession number JN041560), Nocardioid asiatica (n=2; Genbank accession number JN041512), Nocardioid farcinica (n=3; Genbank accession number JN041682), Nocardioid carnea (n=2; Genbank accession number JN041599) and one each of Nocardioid asistata, Nocardioid crostossepae and Nocardioid testacea (Genbank accession numbers JN041487, AY756548 and AB192415, respectively).

Clinical features
Demographic data and information pertaining to the source of isolation, as well as the clinical symptoms of the patients yielding these isolates, are provided in Table 1. Thirteen patients (seven male and six female, 17 to 73 years of age) were diagnosed as having Nocardioid infections. Five patients were immunocompromised, and eight were apparently immunocompetent with predisposing factors such as cystic


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<table>
<thead>
<tr>
<th>Case</th>
<th>Age (years)</th>
<th>Patient origin</th>
<th>Clinical diagnosis</th>
<th>Clinical specimen</th>
<th>16S rRNA identification (genus Nocardia)</th>
<th>Treatment</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>1</td>
<td>42/F</td>
<td>Qatar</td>
<td>Non-Hodgkin’s lymphoma, abscess on back at L1 level</td>
<td>Pus aspirate</td>
<td>N cyriacigeorgica</td>
<td>Ceftriaxone + SXT</td>
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<tr>
<td>2</td>
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<td>India</td>
<td>Chest pain, pleural effusion, lingual infiltrates</td>
<td>Sputum</td>
<td>N cyriacigeorgica</td>
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<tr>
<td>3</td>
<td>36/M</td>
<td>India</td>
<td>Pulmonary infection mimicking tuberculosis</td>
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<td>N cyriacigeorgica</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
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<td>India</td>
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<tr>
<td>5</td>
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<td>Qatar</td>
<td>Cystic fibrosis with pneumonia</td>
<td>BAL</td>
<td>N otitidiscaviarum</td>
<td>Clarithromycin + SXT, moxifloxacin</td>
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<tr>
<td>6</td>
<td>50/F</td>
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<tr>
<td>7</td>
<td>68/M</td>
<td>Qatar</td>
<td>Renal transplant</td>
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<td>N farcinica</td>
<td>Meropenem + SXT</td>
<td>Recovered</td>
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<td>8</td>
<td>50/F</td>
<td>India</td>
<td>Breast cancer on chemotherapy with central line-related sepsis</td>
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<tr>
<td>9</td>
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<td>Pulmonary tuberculosis, bronchiectasis</td>
<td>Sputum</td>
<td>N canea</td>
<td>Anti-TB</td>
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<tr>
<td>10</td>
<td>36/M</td>
<td>Egypt</td>
<td>Pulmonary infection mimicking tuberculosis</td>
<td>Sputum</td>
<td>N canea</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>11</td>
<td>73/F</td>
<td>Qatar</td>
<td>Non-Hodgkin’s lymphoma, paravertebral abscess at L3-L5, on chemotherapy</td>
<td>CT-guided aspirated fluid</td>
<td>N crassostreae</td>
<td>Meropenem + SXT</td>
<td>Improved; died from progressive disease</td>
</tr>
<tr>
<td>12</td>
<td>34/M</td>
<td>India</td>
<td>Previous case of pulmonary tuberculosis</td>
<td>Sputum</td>
<td>N testacea</td>
<td>None</td>
<td>NA</td>
</tr>
<tr>
<td>13</td>
<td>44/F</td>
<td>Syria</td>
<td>Breast cancer with pulmonary infection</td>
<td>Sputum</td>
<td>N asiatica</td>
<td>SXT</td>
<td>Recovered</td>
</tr>
</tbody>
</table>

Anti-TB Antituberculosis treatment; BAL Bronchoalveolar lavage; CT Computed tomography; F Female; M Male; NA Not available; rRNA Ribosomal RNA; SXT Trimethoprim-sulfamethoxazole

Invasive infections

Patients with non-Hodgkin’s lymphomas (cases 1 and 11): Case 1 was a female patient with a Nocardia cavitary lesion in the left upper lung lobe who developed an abscess approximately 3.5 cm in diameter with thick wall present at the left lumborum muscle at the level of L1 vertebral body, as shown in magnetic resonance imaging (Figure 1). Under computed tomography (CT) guidance, a large-gauge needle was inserted into the abscess and a small amount of pus was aspirated and sent to the microbiology laboratory for microscopy and culture; the culture grew N cyriacigeorgica. The patient recovered after treatment with ceftriaxone and sulfamethoxazole-trimethoprim (cotrimoxazole) and is still doing well. In the other woman (case 11), a CT scan revealed a large, paravertebral abscess at the L3-L5 level, present in the left psoas muscle and compressing and displacing the left kidney. A catheter was inserted percutaneously into the abscess; more than 50 mL of pus was aspirated and the catheter was left in situ for further drainage. The abscess extended outward and produced another multiloculated abscess (open arrow) in the left lumborum muscle (case 11).

Blood stream infections: The third case of invasive nocardiosis was found in an immunocompromised patient with breast cancer undergoing chemotherapy and is still doing well. The other two patients with breast cancer (case 1 and the one from case 11) were treated with meropenem and trimethoprim-sulfamethoxazole, but died later from progressive disease.

Figure 1) Magnetic resonance image of a patient (case 1) with pulmonary nocardiosis and dissemination to an abscess approximately 3.5 cm in diameter with thick wall (arrow) in the left lumborum muscle at level of L1 vertebral body caused by Nocardia cyriacigeorgica

Figure 2) Computed tomography scan shows a large abscess caused by Nocardia crassostreae, in the left psoas muscle (solid arrow) extending outward and producing a multiloculated abscess (open arrow) in the left lumborum muscle (case 11)
chemotherapy (case 8), who was diagnosed with N. farcinica infection. She was successfully treated with trimethoprim-sulfamethoxazole.

Pulmonary nocardiosis
Pulmonary nocardiosis was diagnosed in eight patients in the present study. In case 3, an immunocompetent male patient with chest pain, pleural effusion and lingular infiltrates, a CT scan showed a small area of consolidation present in the inferior segment of lingula of the left lung, along with ill-defined hazy linear shadowing present at the periphery of the lateral segment of the left lower lobe (Figure 3). Sputum culture grew N. cyriacigeorgica, and the patient recovered after treatment with azithromycin.

Antimicrobial susceptibility
The antimicrobial susceptibility patterns of the 13 Nocardia species for six antibacterial agents are summarized in Table 2. Growth inhibition ellipses were uniform and well delineated, and the points of intersection with the Etest strips were easy to determine, except for N. otitidiscaviarum (case 4), in which the mutant colonies appeared with cotrimoxazole Etest strips at a minimum inhibitory concentration of 12 µg/mL. All Nocardia isolates were susceptible to linezolid, but showed various susceptibility patterns to other antimicrobial agents. The N. croftiae isolate (case 11) was resistant to amikacin, cefotaxime and imipenem. The N. otitidiscaviarum isolate (case 4) was resistant to imipenem, cotrimoxazole and cefotaxime, and intermediate for moxifloxacin. N. otitidiscaviarum isolate (case 5) was resistant to the antimicrobial agents cefotaxime and imipenem. Eight Nocardia isolates showed beta-lactamase activity by nitrocefin disk test. Positive reaction was demonstrated within 5 min. The nitrocefin test was negative for N. carneae, N. testaeae and N. asiatica, whereas N. cyriacigeorgica isolates showed variability in the results of nitrocefin test, with one of the three strains being negative. Positive beta-lactamase Nocardia included N. otitidiscaviarum and N. croftiae, which also exhibited resistance to cefotaxime and imipenem.

DISCUSSION
Identification of Nocardia to species level using phenotyping is difficult (1,2). Sequencing of the 16S rRNA gene enables more accurate identification, and the application of this technique has resulted in the identification of new and clinically important species of Nocardia over the past 10 years (28-30). The number of reported clinical cases caused by opportunistic nocardiosis infections is constantly rising. Seven species of Nocardia from 13 cases were reported in a relatively short period during the present study (Table 1). N. cyriacigeorgica and N. farcinica are the most common species associated with clinical specimens in Qatar, each represented by three cases, collectively constituting 46% of the cases. N. cyriacigeorgica is frequently isolated from clinical specimens (31,32). The most frequently reported cases of N. cyriacigeorgica constitute disseminated infections with various risk factors including bacteremia in a renal transplant (33), brain abscess in HIV (34), endocarditis (35) and pulmonary infections (36). In the present study, N. cyriacigeorgica was isolated from pulmonary infections in two cases and one disseminated infection in a patient with non-Hodgkin’s lymphoma. N. farcinica has been reported to be an increasing cause of localized and disseminated infections in immunocompromised patients in recent years (37-39), but bacteremia remains a rare finding (40,41). In the present study, we reported a case of bacteremia in a 50-year-old woman with breast cancer undergoing chemotherapy. The second case was a pulmonary infection in a renal transplant patient, treated successfully with meropenem/trimethoprim-sulfamethoxazole, whereas the third case was conjunctivitis in a patient with no apparent immune dysfunction. Although Nocardia infection of any type involving the eye is rare, several species have been diagnosed as a cause of keratitis (42); the isolation of N. farcinica in the present study will be added as a possible etiological agent of eye infection. N. otitidiscaviarum has been isolated from a fatal brain abscess in a patient with chronic obstructive pulmonary disease (22), in a case of bacteremia (43) and also from pulmonary infection (44); in the present study, this species was found in two cases: corneal abscess in an immunocompetent patient and severe pneumonia in a patient with cystic fibrosis. In a retrospective analysis that included 17 cystic fibrosis patients (18), five Nocardia species (including N. otitidiscaviarum) were considered as colonizers and oral antibiotic therapy did not appear to affect the clinical outcome. A patient with cystic fibrosis in the present study (case 5) was hospitalized for severe pneumonia that was treated successfully with clarithromycin, trimethoprim-sulfamethoxazole and moxifloxacin with clinical improvement and negative post-treatment sputum culture.

Several rare pathogens were identified in the present study. N. carneae, less frequently encountered as a human pathogen (32), was isolated from two cases of pulmonary infections. In one report, the species was isolated from a pulmonary infection in a patient with tuberculosis (45). To our knowledge, N. croftiae has not been reported as a human pathogen since its isolation in 1998 from Pacific oysters (46). This species was isolated from a paravertebral abscess at L3-L5 from a patient with a non-Hodgkin’s lymphoma undergoing chemotherapy. N. testaeae, isolated from sputum of a patient with previous pulmonary tuberculosis in the present study, has been rarely isolated from clinical specimens (47). N. asiatica, a rare agent of
Nocardiosis, was first described in 2004, including five strains isolated from Asia (48), and six documented clinical isolates causing pneumonia or cutaneous infections in patients with HIV and a bone marrow recipient (32), and a disseminated infection in HIV patient (31). It was isolated in the present study from a breast cancer patient with pulmonary infection (case 13). This suggests that, although rarely seen in clinical specimens, *N asiatica* is associated with immune dysfunction. All patients were treated with appropriate antibiotics and the infection resolved without further sequelae.

The organisms are readily aerosolized with dust and the respiratory tract remains the main portal of entry, with the majority of patients presenting with pulmonary involvement (4,49). Due to its nontypical manifestations, nocardiosis is frequently misdiagnosed; the initial diagnosis is often pneumonia, tuberculosis or lung abscesses. Radiographic presentation may reveal bronchiectasis with pneumonia (Figure 3).

Five immunocompromised patients (Table 1; cases 1, 7, 8, 11 and 13) were successfully treated with the appropriate antibiotics, but case 11 died from a progressive hematological disease. Disseminated nocardiosis, particularly in those with central nervous system involvement or bacteremia, has a poor prognosis with a high mortality rate in immunocompromised hosts (6,17,22,50).

Accurate identification of *Nocardia* species is important because different species may have different antimicrobial susceptibilities. Linezolid had a distinctive activity pattern against all *Nocardia* species (Table 2); these results are in accordance with previously reported susceptibility patterns for linezolid (51-53). The data support linezolid as an alternative for the treatment of nocardiosis. All but one isolate of *N otitidiscaviarum* isolates were susceptible to trimethoprim-sulfamethoxazole. Susceptibility of *Nocardia* species to trimethoprim-sulfamethoxazole is variable; it was reported that one strain was intermediately susceptible to cefotaxime and imipenem. One strain was intermediately susceptible to cefotaxime. Resistance to cefotaxime and imipenem does not appear to be mediated by beta-lactamase, but rather by decreased affinities of penicillin binding-proteins for these molecules (35).

Among other species, isolates of the *N asiatica*, *N carneae* and *N taeae* have been reported only rarely as human pathogens. *N asiatica* was resistant to moxifloxacin but susceptible to all other five tested antimicrobial agents. However, few isolates were tested for susceptibility, which showed a resistance pattern for ciprofloxacin (51,53), suggestive of a quinolone resistance profile. This class of antibiotics may not be considered for the treatment of infections caused by this species.

The present study is the first report of series of *Nocardia* infection, documenting the species prevalent in Qatar. *N cassioceae* was reported for the first time as a human pathogen. The detection of multidrug resistance species necessitate molecular identification and susceptibility testing, and should be performed for all *Nocardia* infections. The disease manifests with different clinical features depending on the *Nocardia* species and underlying conditions. Most patients recovered with combined antimicrobial agents. Trimethoprim-sulfamethoxazole alone or in combination, or sequential with other agents, was effective in treating the majority of patients.

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