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MOLECULAR IDENTIFICATION AND ANTIMICROBIAL RESISTANCE PATTERN OF SEVEN CLINICAL ISOLATES OF *Nocardia* spp. IN BRAZIL

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SUMMARY

Nocardia is a ubiquitous microorganism related to pyogranulomatous infection, which is difficult to treat in humans and animals. The occurrence of the disease is on the rise in many countries due to an increase in immunosuppressive diseases and treatments. This report of cases from Brazil presents the genotypic characterization and the antimicrobial susceptibility pattern using the disk-diffusion method and inhibitory minimal concentration with E-test® strips. In summary, this report focuses on infections in young adult men, of which three cases were cutaneous, two pulmonary, one neurological and one systemic. The pulmonary, neurological and systemic cases were attributed to immunosuppressive diseases or treatments. Sequencing analysis of the 16S rRNA segments (1491 bp) identified four isolates of *Nocardia farcinica*, two isolates of *Nocardia nova* and one isolate of *Nocardia asiatica*. *N. farcinica* was involved in two cutaneous, one systemic and other pulmonary cases; *N. nova* was involved in one neurological and one pulmonary case; and *Nocardia asiatica* in one cutaneous case. The disk-diffusion antimicrobial susceptibility test showed that the most effective antimicrobials were amikacin (100%), amoxicillin/clavulanate (100%), cephalexin (100%) and ceftiofur (100%), while isolates had presented most resistance to gentamicin (43%), sulfamethoxazole/trimethoprim (43%) and ampicillin (29%). However, on the inhibitory minimal concentration test (MIC test), only one of the four isolates of *Nocardia farcinica* was resistant to sulfamethoxazole/trimethoprim.

KEYWORDS: Nocardiosis; *Nocardia;* Opportunistic disease; Antimicrobial susceptibility test.

INTRODUCTION

Nocardiosis is a chronic and severe pyogranulomatous disease caused by the environmentally ubiquitous actinomycete of the *Nocardia* genus[1]. Nocardiosis is an emerging disease in humans and animals worldwide[2,13,26,32,34]. According to BAIO *et al.* (2013) nocardiosis is a neglected disease, particularly in patients with some degree of immunosuppression[1]. For many years, since Edmond Nocard's first description of the pathogen in 1888, its diagnosis was based on phenotypic methods[17]. To date, 102 species of *Nocardia* have been discovered using molecular methods, of which seven have been reclassified and 90 currently stand on the list of prokaryotic names with standing nomenclature[24]. Among these species, at least 25 are pathogenic to humans and animals[8,13,17,29]. *N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova*, *N. cyriacigeorgica* and *N. veterana* are the main species related to nocardiosis in humans[1,11,14,17].

Either tegumentary injury or the inhalation of bacteria is considered the most common route of transmission of *Nocardia* spp. in humans[7]. Clinically, the main manifestations of human nocardiosis are pneumonia, encephalitis, lymphadenitis, lymphangitis and cutaneous tissue lesions[33].

Therapy consists of a prolonged course, its success depending on the species of bacteria, the virulence of the strain, the organs affected, the time of evolution and the health status of the susceptible individual(s)[1]. *Nocardia* spp. is refractory to conventional antimicrobial therapy. Sulfonamides potentiated by trimethoprim, minocycline, aminoglycosides (amikacin, gentamicin) and cephalosporins (ceftiofur, ceftriaxone, cephalexin) alone or in combination, based on *in vitro* tests[3], are the choices of treatment for human and animal nocardiosis[26,28,31].

The purpose of this case report is to present the species of *Nocardia* involved in human nocardiosis in Brazil, and their respective drug susceptibility pattern.

MATERIAL AND METHODS

Isolates. Seven strains of *Nocardia* spp., identified in three Hospitals from different states of Brazil (one in Rio Grande do Sul, one in São Paulo and five in Paraná), were isolated from clinical cases of nocardiosis. The strains were isolated from different specimens (bronchial wash, cutaneous and organ fragments) in defibrinated sheep blood agar (5%), Sabouraud or Lowenstein agar, and maintained aerobically at 37 ºC for three to

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10 days. Colonies suggestive of the genus *Nocardia* were evaluated by dry, convex, adherent, and white to orange color aspects. After 48 to 72 hours post-inoculation, colonies were submitted to Gram and Kinyoun stain[8,27]. Gram-positive, filamentous to cocobacillary, partially acid-fast organisms were identified as *Nocardia*.

Molecular identification. Molecular analysis was carried out in the Medical Mycology Research Center, Chiba, Japan. Genomic DNA for sequencing was performed according to KAGEYAMA *et al.,* 2004. The 16S rRNA gene was amplified by PCR using a DNA thermal cycler (TaKaRa Bio Inc., Chiba, Japan) under the following conditions: 35 cycles at 94 ºC for 60 seconds for denaturation, 60 ºC for 60 seconds for primer annealing, and 72 ºC for 120 seconds for primer extension. PCR primers were the prokaryotic 16S rRNA universal primer pairs, 8F and 691R, 520F and 1100R, and 926F and 1542R. DNA sequences were determined with an automatic sequence analyzer (ABI PRISMTM 3130; Applied Biosystems, Japan), using the same primers and a dye terminator cycle sequencing kit (Applied Biosystems). Near-complete 16S rRNA gene sequences consisting of approximately 1400 bases pairs were obtained. The sequence of the 16S rRNA gene was compared to the GenBank database using BLAST, and 16S rRNA sequences of related *Nocardia*-type strains were retrieved from the database. These sequences were submitted to GenBank/JJBJ/EMBL.

***In vitro* drug susceptibility tests.** All strains were examined using the disk-diffusion test and minimum inhibitory concentration test (MIC test) based on E-test™ (E-test™, AB biodisk, BioMérieux, Dalvägen, Sweden), according to the procedures described by GLUPCKZYNSKI *et al.* 2006 and NCCLS 2006. Isolates resistant to three or more antimicrobials were considered multi-resistant[23].

The study of drug susceptibility in Actinomycetes is more laborious because this group of bacteria usually grows in clumps. Due to this adherent characteristic during bacterial growth, the observed irregular broth turbidity makes it difficult to measure the optimum inoculum concentration. Naturally, the precise concentration of unit colony formation is essential for the correct interpretation of both antimicrobial tests[11,20].

The isolates were briefly subcultured twice in blood agar (5%) to ensure their purity. After 48 hours, they were inoculated in brain-heart-infusion at 37 ºC for another 48 hours. Sterile glass beads were added at the time the strains were vortexed to decrease the formation of clumps and subsequently obtain a more accurate optical density (OD) equivalent to a 0.5 McFarland standard to disk-diffusion test and 1.0 McFarland standard for E-test.

All of the strains were submitted to a disk-diffusion test and the inhibition zones interpreted following standards according to BAUER *et al.,* 1966 and AMBAYE *et al.* 1997. The antibiotics selected were amikacin (30 µg), ampicillin (10 µg), amoxicillin/clavulanate (20 µg/10 µg), ceftiofur (30 µg), cefoperazone (75 µg), ceftriaxone (30 µg), cephalexin (30 µg), cefuroxime (30 µg), cefalonium (30 µg), imipenem (10 µg), gentamicin (10 µg), mynocicline (30 µg) and sulfamethoxazole/trimethoprim (25 µg).

In the MIC test, a maximum of five E-test strips were attached to Mueller Hinton agar and were then incubated at 37 ºC. The results were recorded after 48 - 72 hours because of the organisms' fastidious growth[3,20]. The following antibiotics were used: amikacin (0.016 - 256 µg/mL), ampicillin (0.016 - 256 µg/mL), amoxicillin/clavulanate (0.016 - 256 µg/mL), ceftriaxone (0.016 - 256 µg/mL), gentamicin, sulfamethoxazole-trimethoprim (0.002 - 32 µg/mL) and imipenem (0.002 - 32 µg/mL).

The similarities between the results from the disk-diffusion test and E-test were analyzed using the Kappa agreement index. According to the values obtained, the agreement analysis between the tests followed the criteria established by THRUSFIELD (1995). All statistical analysis was done using Bioestat v.5.0 and SPSS v.14 packages. The Chi-squared and Fisher's exact tests were used to analyze whether the resistance percentages of *Nocardia* spp. were normally distributed between strains for each antibiotic tested with the disk-diffusion method. The level of significance for this test was $p < 0.05$[33].

RESULTS

All strains were taken from young adult males between 28 and 35 years of age. Three patients displayed cutaneous manifestations (exhibiting fistulous mycetomas due to traumatic inoculation), while two showed pulmonary, one neurological and one systemic. Two individuals with pulmonary symptoms, one with neurological, and one with systemic were co-infected with immunosuppressive diseases, or were undergoing/had undergone prolonged treatments with chemotherapy or corticotherapy. Of all the patients, four died due to the severity of their disease (these were pulmonary, neurological and systemic cases), two recovered (cutaneous manifestations), and one case had no documented outcome (cutaneous manifestation) [Table 1].

The microbiological culture of samples showed dry, convex, strongly adherent, whitish to orange-brown colonies with a powdery surface after 48-96 hours of incubation at 37 ºC. Gram and modified Ziehl-Neelsen stains showed thin, branched filaments sometimes fragmented in cocobacillary forms, suggestive of the *Nocardia* genus.

The sequence of the 16S rDNA gene enabled the identification of four strains - *N. farcinica*, two *N. nova*, and one *N. asiatica* - based on its 99.6% or higher sequence similarity to the reference sequence in GenBank (DDBJ/GenBank/EMBL), using BLAST as recommended by CLSI, 2008. The access number for the isolates in GenBank is as follows: IFM 11128/ AB 633331 - *N. nova;* IFM 11096/ AB 630965 - *N. farcinica;* IFM 11231/ AB 636474 - *N. farcinica;* IFM 11232/ AB 636475 - *N. farcinica;* IFM 11099/ AB 630966 - *N. nova;* IFM 11100/ AB 630967 - *N. asiatica;* IFM 11285/ AB 638765 - *N. farcinica*.

In this study, *N. farcinica* was observed in two cutaneous, one systemic and other pulmonary cases; *N. nova* was present in one neurological manifestation and one pulmonary; and *Nocardia asiatica* in one cutaneous case (Table 1).

The *in vitro* drug susceptibility test, based on disk-diffusion, is presented in Table 2. Based on the disk-diffusion test, *Nocardia* spp. isolates were sensitive to amikacin, amoxicillin/clavulanate, cephalexin, cefalonium, ceftiofur, ceftriaxone and minocycline. However, around 50% of the *Nocardia* spp. isolates were resistant to ampicillin, gentamicin and sulfamethoxazole/trimethoprim. Of the seven isolates, two were

Table 1
Summary of clinical presentation and outcomes of seven patients with nocardiosis. Santa Maria, RS; Curitiba, PR and Botucatu, SP, Brazil

Nocardia species	Clinical presentation	Site of isolate	Age/sex	Underlying condition	Treatment	Outcome
N. nova	Neurologic	Liquor	32/male	Chemotherapy	NA*$_1$	Death
	Pulmonary	Sputum	33/male	HIV	4 drugs*$_2$	Death
N. farcinica	Dermatologic	Biopsy	30/male	None	NA	Recovery
	Dermatologic	Biopsy	28/male	None	NA	Recovery
	Pulmonary	Broncoalveolar wash	35/male	HIV	NA	Death
	Systemic	Liquor	34/male	Chemoterapy	NA	Death
N. asiatica	Dermatologic	Biopsy	35/male	NA	NA	NA

*$_1$ NA = not available; *$_2$ Patient came in on advanced stage of disease, he was treated with clindamycin, sulfamethoxazole/trimethoprim, imipenem and amphotericin before death.

multi-resistant to three or more antimicrobials. One isolate of *N. farcinica* was resistant to ampicillin, cefoperazone and gentamicin; and the other isolate of *N. farcinica* was resistant to ampicillin, sulfamethoxazole/trimethoprim, gentamicin and cefuroxime.

The minimum inhibitory concentration of tested antimicrobials showed suitable breakpoints, with all the isolates being susceptible to the tested antimicrobials (Table 3) except for one isolate of *Nocardia farcinica,* which was resistant to sulfamethoxazole/trimethoprim.

Table 2
Standard inhibition zone diameter and percentage of susceptibility of 7 *Nocardia* spp. isolates to selected antimicrobials in disk-diffusion test. UNESP, Botucatu, SP, Brazil

Antimicrobial	Zone diameter (mm)[a]			% Susceptible
	R	I	S	
Amikacin	≤14	15-16	≥17	100
Amoxicillin/ clavulanate	≤13	14-17	≤13	100
Ampicillin	≤13	14-16	≥17	29
Cephalexin	≤14	15-17	≥18	100
Cephalonium	≤14	15-17	≥18	100
Cefoperazone	≤15	16-20	≥21	43
Ceftiofur	≤17	18-20	≥21	100
Ceftriaxone	≤13	14-20	≥21	86
Cefuroxime	≤14	15-17	≥18	72
Imipenem	≤13	14-15	≥16	72
Gentamicin	≤10	11-14	≥15	57
Mynocicline	≤14	15-19	≥19	100
Sulfamethoxazole/ trimethoprim	≤10	11-15	≥16	43

[a] Zone of inhibition diameter (mm) by disk diffusion method susceptibility interpretative guidelines based on NCCLS (2006), AMBAYE *et al.* (1997), and BAUER *et al.* (1966). R = resistant, I = intermediate, S = susceptible.

Table 3
Minimum inhibitory concentrations (μg/mL) and susceptibility proportion estimates of *Nocardia* spp. isolated from seven case reports. UNESP, Botucatu, SP, Brazil

Antimicrobials	MIC_{50}[a] (μg/mL)	MIC_{90}[a] (μg/mL)	%Susceptible (Overall n=7)
Amikacin	0.20	0.40	100
Amoxicillin/ clavulanate	0.5	3	100
Ampicillin	0.25	1	100
Cephalexin	1	2	100
Ceftriaxone	1.5	2	100
Gentamicin	1	2	100
Imipenem	0.75	1,5	100
Sulfamethoxazole/ trimethoprim	1.5	3	86

[a] MIC_{50} and MIC_{90}: values are concentrations at which ≥ 50% and ≥90% of isolates are inhibited by antimicrobials.

The agreement between the tests was considered low by the statistical analysis.

DISCUSSION

These findings reinforce that molecular techniques are a reliable, suitable and quick method for the diagnosis of species of *Nocardia* genus taken from a human origin. Phenotypic evaluations could be performed to identify the *Nocardia* species, combining tests such as the hydrolysis of organic compound (adenine, xanthine, hypoxanthine, casein, esculin and tyrosine), carbohydrate assimilation, antimicrobial susceptibility pattern, citrate utilization and acetamid and arylsulfatase utilization, among others[8,27,36]. However, they are usually laborious, time-consuming, and require experience in evaluating the results. For this reason, molecular methods emerged as an alternative that can be used on several different

body fluids and tissue samples, and can also be used for identifying strains that are difficult to grow in a conventional medium[8,29].

The 16S rRNA gene is highly conserved with constant regions and, to date, its complete sequence of approximately 1400 bp has a considerably large database on GenBank, which allows for the identification of most *Nocardia* species[29]. However, because 16S rRNA shows minimal variation and is also present in different numbers of copy variants, this gene should be sequenced in its total 1400 bps and follow the standards of CLSI, which recommends a similarity higher than 99.6%. Nowadays, other researchers are reporting on the sequencing of housekeeping genes with a better discriminatory power, even with partial gene sequencing[16,29].

The incidence of human nocardiosis has increased in the last two decades across several countries, particularly in patients affected by immunosuppressive therapies or diseases[1,8]. In Brazil, the 22 cases of human nocardiosis, predominantly displaying pulmonary symptoms, were most prevalent in adult males (59.2%) between the ages of 21 and 84. Most cases were related to immunosuppressed conditions (69.9%), such as transplants, corticotherapy and being HIV-positive[30]. These findings agree with similar observations that described immunocompromised conditions in humans affected by nocardiosis[10,19,21,30], reinforcing the opportunistic behavior of the *Nocardia* genus[7]. However, it is important to stress that cutaneous presentations are not always associated to a previous health condition, indicating possible transmission through cutaneous trauma, as suggested by other studies[4]. In this study, nocardiosis only affected men between the ages of 28 and 35. This is consistent with the results of other studies, which have also identified similar occurrences of nocardiosis in young adult males, indicating the occupational risk of human infection by *Nocardia* species in mainly immunocompromised patients exposed to the agent within their environment[10,21,34].

The clinical picture of human nocardiosis is diverse, though cutaneous and pulmonary manifestations are present in the majority of cases[4,5]. In the last few years, *N. farcinica* has been the most isolated species in reviews of different kinds of patient infection, whether with or without immunosuppression, representing around 22% and 45% of cases respectively[19,26,32,34]. Evidence shows that *N. farcinica* is widely distributed in the environment, has a high potential of virulence and is closely associated to a great number of fatal nocardiosis cases, including those with systemic dissemination[8]. Cases related to *N. farcinica* confirmed the severity of the disease in immunosuppressed patients.

In other reports, *N. nova* has been associated to approximately 20% of isolates in the United States and was the most pathogenic species in Canada until 2008[34,35]. *N. nova* has been attributed to different clinical presentations in humans, mainly related to immunosuppressive conditions or trauma[4,35]. These findings reinforce this species' pathogenic potential for humans, and the risks of developing systemic nocardiosis, resulting in death.

N. asiatica was recently identified in human nocardiosis in Brazil, and this is one of few reports worldwide[5,21,22] which reinforces the necessity of identifying more isolates and understanding their epidemiology.

The lower efficacy of human strains to gentamicin and ampicillin in the disk- diffusion test is probably related to the pattern of low susceptibility of *N. farcinica* resistance, indicating that these are not recommended as therapy[20,31]. However, the high efficacy of cephalosporins against *N. farcinica* isolates and the high success rate of amoxicillin/clavulanate against *N. nova* could be justified by the variability in β-lactamase activity present in the bacteria's cell wall[3,20]. In agreement with previous results, minocycline appeared to be highly effective as *in vitro* against the *Nocardia* species isolated in these reports[3,31], however MUÑOZ *et al.* (2007) observed resistance to this drug in *N. nova* and *N. cyriacigeorgica*.

Sulfamethoxazole/trimethoprim presented a lower efficacy in disk-diffusion tests against the isolates and are perhaps, therefore, not appropriate for therapy. Similar observations with a larger number of clinical isolates were made by TREMBLAY *et al.* (2011), informing of the importance of establishing treatment protocols for *in vitro* tests. However, despite the worldwide resistance pattern to sulfonamides recorded in clinical isolates, few patients failed therapy with these drugs, suggesting that the inhibition breakpoint for sulfonamides still poses a challenge and varies among different laboratories[9]. This can result from the methodology suggested by CLSI in which sulfonamide breakpoints are based on 80% of inhibition of growth endpoint compared to the 100% inhibition of growth by other drugs. In future research, more isolates should have the antimicrobial profile analyzed using the same CLSI specifications, as performed in this study, and new breakpoints for *Nocardia* species should perhaps be considered. Moreover, the indiscriminate use of the drug for comorbidities can induce resistance of *Nocardia* to sulfonamides and molecular mechanisms, thus it should be analyzed[8,9,26].

Some studies showed the applicability of the E-test for antimicrobial susceptibility testing for the analysis of actinomycete resistance[20]. However, disagreement between MIC and disk diffusion tests has been indicated in other papers[3,25]. These differences probably occur due to colony lumps formed during culture growth, which makes it difficult to obtain a precise McFarland scale, or an ideal count of colony-forming units[25]. So far, for *Nocardia* the broth dilution method is still favorable, in comparison to the E-test, in order to assure the significance of the minimum inhibitory concentration method.

Despite the small number of isolates, it was possible to notice that nocardiosis in Brazil mainly affects men and immunosuppressed patients with localized or disseminated infection. Clinical manifestations could vary depending on the species, virulence of isolates, and immunocompromised factors of the patients. The cases reported in this study were seen in patients with a higher comorbidity, predominantly associated with pulmonary or disseminated forms. Antimicrobial resistances of isolates reinforce the importance of prior *in vitro* tests before initiating therapy. Further investigation with a larger number of cases and isolates is necessary.

RESUMO

Identificação molecular e perfil de sensibilidade a antimicrobianos de sete isolados clínicos de *Nocardia* spp. no Brasil

Nocardia é um microorganismo ubiquitário relacionado a infecções piogranulomatosas, com difícil resolução tecidual em humanos e animais. A doença é mundialmente emergente devido ao aumento de doenças e tratamentos imunossupressores. Este relato de casos ocorridos no Brasil visa apresentar a identificação molecular dos isolados e o padrão

de sensibilidade a antimicrobianos por disco-difusão e concentração inibitória mínima (CIM) através de fitas E-test®. Os casos ocorreram em homens, em idade adulta. Três quadros foram cutâneos, dois pulmonares, um neurológico e um sistêmico. O quadro respiratório, o neurológico e um sistêmico estavam associados à doença ou terapia imunossupressoras. O sequenciamento do gene 16S rRNA (1491pb) possibilitou a identificação de quatro isolados de *Nocardia farcinica*, dois de *Nocardia nova* e um de *Nocardia asiatica*. *N. farcinica* foi observada em dois casos dermatológicos, um pulmonar e um quadro sistêmico, *N. nova* foi isolada de um caso neurológico e outro pulmonar; e *N. asiatica* em um caso dermatológico. O teste de disco-difusão mostrou que amicacina (100%), amoxicilina/clavulanato (100%), cefalexina (100%) e ceftiofur (100%) foram mais efetivos; enquanto gentamicina (43%), sulfametoxazol/trimetoprim (43%) e ampicilina (29%) foram menos efetivos. No entanto, no teste de concentração inibitória mínima (CIM), apenas um dos quatro isolados da espécie *Nocardia farcinica* mostrou-se resistente a sulfametoxazole-trimetropina.

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