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PCR-RFLP Methodology to Identify *Nocardia* Isolates in Cuba

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Abstract

Nocardiosis diagnosis is a major challenge. The clinical features and radiological findings are nonspecific. Traditionally, *Nocardia* identification is based on colonial and microscopical morphology and biochemical tests. However, molecular biology techniques allow a better characterization of species and biotypes. PCR-RFLP of the 65-kDa heat shock protein (HSP) gene provides a rapid, sensitive, and time and labor-efficient method for this proposal. Using this technique, six of eight isolates tested were identified as *Nocardia* asteroides type VI. PCR-RFLP of the 65-kDa HSP gene could be very useful for determining the incidence of this pathogen in different population groups and its association with susceptibility/resistance profiles to the drugs of choice for treatment. This work is the first molecular detection of *Nocardia* species in Cuba.

Keywords

***Nocardia*, PCR-RFLP, Cuba, Molecular Identification, 65-kDa HSP Gene**

1. Introduction

The term *Nocardia* currently refers to anaerobic, filamentous, Gram-positive genus of bacteria that is found mainly in water and soil. The genus belongs to the *Actinomycetales* order and the *Nocardiaceae* family. Approximately 100 different species have been reported according to recent taxonomic studies (http://www.bacterio.cict.fr/n/nocardia.html) [1]. In 1891, the first case of human nocardiosis was described in a

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patient with pseudotuberculosis syndrome and brain abscesses. Since then, approximately 30 species have been recognized as human pathogens [2].

The incidence of nocardiosis varies geographically according to several factors, such as cases of human immunodeficiency virus infection, transplants, and neoplastic and rheumatic diseases as well as climate, socioeconomic conditions and laboratory procedures for *Nocardia* identification [3]. Although cases of infection have been reported in the central nervous system, skin, and eyes (keratitis and endophthalmitis), pulmonary disease is usually the most characteristic and frequent clinical manifestation of nocardiosis [2].

Nocardiosis diagnosis is a major challenge. The clinical features and radiological findings presented by nocardiosis patients are nonspecific. The features can be confused with other pulmonary infections with different etiologies [2]. The serological techniques tested to date are not sufficiently sensitive, and these techniques present a large number of cross-reactions with other actinomycetes. In addition, laboratory diagnosis is based on microscopy and culture methods in which the results might lead to significant errors due to the confusion of the examined sample with rapidly growing mycobacteria. With the advent of molecular techniques and their involvement in the current taxonomic changes, phenotypic methods have shown significant limitations and inconsistent results for the identification of *Nocardia* species [1].

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of the 65-kDa heat shock protein (HSP) gene provides a rapid, sensitive, and time and labor-efficient method for characterization of species and biotypes of *Nocardia.* In addition, this methodology has provided reliable information to better understand the epidemiology of the infections produced by *Nocardia* genus [4].

Nocardia sp. are also responsible for actinomycetoma, an anatomo-clinical syndrome characterized by a volume increase, deformation of the area, and multiple nodules that drain filamenting exudates, in which the bacteria are forming grains. Rapid identification of the causative agent in these cases is very important due to the differentiation of actinomycetoma from eumycetomas caused by fungi; in both cases the therapeutic management is completely different [5].

Our aim in the present preliminary study was to identify *Nocardia* isolates by PCR-RFLP of the 65-kDa HSP gene in Cuba.

2. Material and Methods

2.1. Study Samples

The present study included eight clinical isolates of *Nocardia* that were identified previously by colony morphology (pigment production and colony aspects), microscopic characteristics after Ziehl-Neelsen staining, and resistance to lysozyme [6].

2.2. DNA Extraction

Isolates were grown on Sabouraud agar and a loop of *Nocardia* sp. was suspended in 200 µL of TE (10 mM Tris, 1 mM EDTA [pH 8]). For DNA isolation from *Nocardia*, QIAGEN DNA Mini kit (QIAGEN, Valencia, CA) was used. In brief, 200 µl of enzymatic lysis buffer was added to the initial mix and incubated at 37˚C for 1 hour. A 25 µl aliquot of a proteinase K (QIAGEN, Valencia, CA) and 200 µl of AL buffer were added and incubated at 56˚C for 1 hour. 200 µl of 100% ethanol was added to the sample and mixed by vortexing. The mixture was then transferred a QIAmp Mini Spin column with silica membrane for the adsorption of bacterial DNA. After, samples were washed with 500 µl of AW1 and AW2 buffer respectively as described the manufacturer. Finally, the DNA extracted was eluted using 120 µl of the AE buffer.

2.3. PCR Amplification

A 439-bp segment of the 65-kDa HSP gene was amplified in 50 µL PCR reaction volumes containing 15 µL 2× HotStarTaq Plus mastermix (QIAGEN, Hilden, Germany); 2 mM $MgCl_2$, 0.5 µL of each primer (TB11 (5'-ACC AACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3')) and 5 µL of extracted DNA. The reaction was subjected to 40 cycles of amplification (94˚C, 60˚C, and 72˚C for 1 min at each temperature, and then for a 10-min extension period at 72˚C) [4]. PCR products were visualized by agarose gel electrophoresis with etidium bromide (10 mg/ml) under UV light.

2.4. RFLP Analysis

For restriction endonucleases analysis (RFLP), the details of reaction system for each enzyme in RFLP analysis were described in [**Table 1**]. Enzymes and buffers were purchased from NEW ENGLAND, BioLabs® Inc. Restriction fragments were electrophoresed on 3% NuSieve® 3:1 Agarose (Lonza, USA).

2.5. Sequencing

Amplicons from all samples that yielded positive PCR results were purified using a QIAquick PCR Purification kit (QIAGEN, Hilden, Germany) and sequenced directly from both ends by using a model ABI 377 automated sequencer and an ABI prism Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Consensus sequences were edited manually, analyzed and aligned using ChromasPro (version 1.7.4, Technelysium Pty Ltd., Australia), BLAST software accessible online (www.ncbi.nlm.nih.gov), and CLUSTAL X (version 2.1). Sequences were compared with *Nocardia* reference gene sequences.

3. Results and Discussion

Infections caused by *Nocardia* sp are uncommon in humans, but occur with some frequency in patients who are severely immunocompromised. Among *Nocardia* species, at least 25 are pathogenic to humans and animals. *N. asteroides*, *N. brasiliensis*, *N. farcinica*, *N. nova*, *N. cyriacigeorgica* and *N. veteran* are the species mainly described [7]. With different prevalence *N. asteroides* was the predominant species identified in USA, Brazil, France, Japan and Pakistan [8]-[12].

In Cuba, very few studies have addressed the identification of *Nocardia* by molecular methods [13]. According to the results in the scientific literature, we have found only one study that analyzed *Nocardia* identification [13]. Recently, our research group described the amplification of a 439-bp fragment of HSP 65 kDa gene of the genus *Mycobacterium* and described its subsequent restriction endonuclease analysis [14]. Using this technique, six of eight isolates tested (previously described as *Nocardia* by phenotypic methods) were identified as *Nocardia asteroides* type VI based on the RFLP patterns generated with the enzymes that constituted the genotyping algorithm (*Msp* I, *Bsa* HI, *Hinf* I and *Bst* EII) [**Table 2**].

Nocardia asteroides complex comprises at least three different species: *N. asteroides*, *N. farcinica*, and *N. nova.* PCR-RFLP algorithm of HSP 65 kDa gene clearly differentiates *N. asteroids* complex of other *Nocardia* species. Steingrube and colleagues using this methodology demonstrated that *N. asteroides* type VI was the most frequent (47%) in nineteen reference and 156 clinical strains of the genus *Nocardia* belonging to 12 taxonomic

Table 1. Characteristics of reaction system for each enzyme used in RFLP analysis.

Enzymes used	Reaction system	Reaction temperature	Incubation time
Msp I	PCR reaction mixture—10 µL Nuclease-free water—10 µL 1× CutSmart™ Buffer—2.5 µL *Msp* I (5U)—0.5 µL	37˚C	1 hour
Bsa HI	PCR reaction mixture—10 µL Nuclease-free water—10 µL 1× NE Buffer 4—2.5 µL *Bsa* HI (5U)—0.5 µL	60˚C	1 hour
Hinf I	PCR reaction mixture—10 µL Nuclease-free water—10 µL 1× NE Buffer 4—2.5 µL *Hinf* I (5U)—0.5 µL	37˚C	1 hour
Bst EII	PCR reaction mixture—10 µL Nuclease-free water—10 µL 1× NE Buffer 3—2.5 µL *Bst* EII (5U)—0.5 µL	60˚C	1 hour

Table 2. Algorithm for the identification of *Nocardia* species by PCR-RFLP.

Enzyme used	RFLP pattern obtained
Msp I	180/145-130/120-115
Bsa HI	270/70/60
Hinf I	440 pb
Bst EII	440 pb

groups [4] [15]. Similar results were described by Wilson and co-workers [16].

In the mid-1990s, restriction pattern analysis of HSP 65 kDa method was routinely used to amplify a 439 pb gene fragment that encodes a protein of 65 kDa for mycobacteria [17] [18]. Besides, the conserved nature of this gene allowed rapid differentiation of clinically significant species and taxa of aerobic actinomycetes with an accuracy of 96.8% by PCR-RFLP analysis [4] [15] [16]. This protocol has additional advantages: it can provide reliable results in 24 - 48 hours after receiving the sample in the laboratory; it uses a unique procedure to discriminate among mycobacteria, *Nocardia*, *Rhodococcus*, and other species involved in human health without subsequent sequencing of the amplicon; and it can be established in a microbiology laboratory as a routine protocol without excessive economic cost [4] [14].

On the other hand, Rodriguez-Navas showed that the "*N. asteroids* type VI" pattern was identical to the pattern obtained for *N. abscessus*, *N. asteroids* (ATCC 19247T), *N. brevicatena*, *N. cyriacigeorgica*, *N. paucivorans*, and *N. vinacea*. Thus, the use of PRA can lead to erroneous species identification of both clinical and environmental *Nocardia* isolates [19]. In our study, the pattern obtained by PCR-RFLP were confirmed by nucleotide sequence analysis of this fragment, which showed 100% homology with sequences of *Nocardia asteroides* retrieved from GenBank (ATCC no. 14759). However, new investigations that involve multiple samples (clinical and environmental) from *Nocardia* sp. are necessary.

Molecular methods based on sequencing of the 16 S rRNA and the *gyrB* gene are crucial to *Nocardia* spp. identification and have become the "gold standard" for the identification of *Nocardia* isolates to the species level [11] [20]. However, some species of *Nocardia* have identical 16 S sequences it is limit the differentiation *Nocardia* species. In addition, the sequencing is labor-intensive and difficult to implement for routine use in many clinical laboratory. In contrast, the *Nocardia* HSP 65 kDa gene is less conserved than the 16 S rRNA gene [19].

A rapid and accurate identification of *Nocardia* species is of extraordinary importance to the medical field. i) The most relevant reasons include the high mortality (more than 40%) when the infection is not suspected, ii) subacute pulmonary nocardios is often mimics other respiratory diseases, such as tuberculosis, pneumocystosis, invasive fungal infections, and malignancy, leading to incorrect treatments and behavior regarding the patient, and iii) different species have different susceptibility patterns to the drug chosen to treat the infection [1]. *Nocardia* taxonomy has been linked to specific patterns of antimicrobial susceptibility ever since the previous work by Wallace and colleagues established the presence of six drug pattern types among the *Nocardia asteroides* species complex [21].

In summary, a laboratory with technological capacity is available. This laboratory has an established standardized protocol that provides a correct and prompt response when nocardiosis is suspected. Furthermore, this technique could be very useful for determining the incidence of this pathogen in different population groups and its association with susceptibility/resistance profiles to the drugs of choice for treatment. Finally, this paper presents the first molecular detection of *Nocardia* species in Cuba.

4. Conclusion

PCR-RFLP of HSP 65 kDa gene appears to be a very rapid method for identification of *Nocardia asteroides* type VI. Other advantages are the simplicity and cost of algorithm of work. Finally, its methodology could be an attractive option for many laboratories of developing countries.

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