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Findings in the Molecular and Proteomic Diagnosis of *Nocardia brasiliensis*

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Abstract

Nowadays, cases of so-called neglected diseases (related to poverty) are increasing. One of them is mycetoma and its variants: Actinomycetoma (caused by bacteria) and eumycetoma (caused by fungi). In the case of actinomycetoma, this disease is often not reported and, as it does not cause pain, people who suffer from it usually go to the doctor when the lesions cause them discomfort when moving or work or social problems. While the diagnosis is usually "easy" since it is mainly based on cultures and stains, many times this is omitted, giving rise to subclinical diagnoses. In this work we strive to make an accurate diagnosis for a patient who suffered from actinomycetoma caused by Nocardia brasilliensis and who was treated with miconazole (an antifungal) for 10 years. After collecting the sample, the corresponding cultures and stains were performed to identify the causal agent, which is Nocardia brasilliensis. Although when talking about the causative agents of actinomycetoma, this aforementioned bacteria is the most studied and reported, we undertook the task of confirming in a more precise way the agent with which we were dealing, for which a procedure was standardized for the identification of the 16S rRNA gene of this bacteria by means of genomic PCR and the realization of the genetic fingerprint mediated by restriction enzymes of Restriction Fragment Length Polymorphism (polymorphisms of the length of restriction fragments) for the 18 strains cultivated in Saburoud Dextrose agar plates. Despite the fact that this gene is present in a huge quantity of bacteria and also that this genus of bacteria has the capacity to mutate certain genes with different organisms that share its environment, such as Aspergillus sp, Fusarium sp, Actinomadura sp, Madrurela sp. This gene does not have homologous regions between the different genera and species that have it, since it has been used for more than 20 years in the identification of this bacteria by PCR with restriction enzymes. In addition, the mutations that could be generated do not occur in the region of this gene since thousands of years have to pass so that they can be expressed and therefore be noticed. This is how, through the extraction, purification, and quantification of the genetic material for its subsequent sequencing with the help of virtual tools such as the BLAST program (Basic Local Alignment Search Tool) that belongs to NCBI, we were able to demonstrate the presence of this 16S rRNA gene in order to have an exact diagnosis of Nocardia brasiliensis.

Keywords: Mycetoma, Actinomycetales, Nocardia Brasiliensis, 16S rRNA Gene, PCR

Introduction

The term actinomycetes comes from the Greek words "aktino" (meaning "ray" or "glow") and "mycete" (meaning "fungus"), which refers to a fungus with filaments arranged in a radial pattern [1]. Actinomycetes are prokaryotic organisms, they lack a nuclear membrane and their genetic material is found free in the cytoplasm. They have a high proportion of the nitrogenous bases guanine (G) and cytosine (C), they have ribosomes, but they lack a Golgi apparatus, endoplasmic reticulum and mitochondria. Their cell membrane does not contain ergosterols, and their cell wall contains muramic acid [2].

Actinomycetes are tiny in size, with a diameter of less than 1 micrometer. They reproduce mainly by binary fission, forming a microsiphonate mycelium (less than 1 micrometer in diameter), which implies that their "filaments" fragment into bacilli or cocci. In addition, they have a remarkable resistance to humidity, drought and high temperatures, adapting to diverse habitats such as soil, lakes, rivers, dry wood and thorns [2]. They frequently interact with other living beings, especially with plants, animals and humans, either as parasites or symbionts, although it is also common to find them in decomposing organic material. Regarding their distribution, the genus *Streptomyces* represents between 70% and 90%, *Nocardia* between 10% and 30%, and *Micromonospora* between 1% and 15% [3].

In Mexico, the most frequent species of actinomycetes is *Nocardia brasiliensis*, which is generally isolated from actinomycotic Mycetomas, which has secondary peculiarities with high interspecies phenotypic similarity in its taxonomic classification, causing it to be little known for almost two hundred years and until recently reclassified [1]. Currently, 119 species of *Nocardia* have been described by their genetic fingerprint. *Nocardia brasiliensis* had initially been described as a homogeneous species in its phenotype, but in recent decades it has been shown that there is within its biochemical profile, its susceptibility to antibiotics and in some nucleotide sequences (genes), a high variability that had not been reported before [1].

The use of molecular techniques, with the use of computer tools (*in silico* study), that can promptly identify these differences at the genetic level could help to expand the knowledge we have about this species, analyze and compare genetic patterns, establish groups that form subspecies or genotypes and explain the high variability observed. Likewise, it is expected that these methods will one day be standardized for the clinical diagnostic laboratory of first and second level of health care. With these considerations, a descriptive and cross-sectional study will be carried out, in which the amplicon for the 16S rRNA gene will be detected from DNA extracted from 18 cultures taken from a primary culture of a chronic mycetoma, all of which had colonies with conclusive characteristics with *Nocardia spp* [3].

Screening of the gene 16S ARNr

Of all the available target genes, 16S rRNA gene sequences are the most widely used and are considered the gold standard for routine identification and phylogenetic analysis of isolates of species of the genus *Nocardia*. The advantages of 16S rRNA gene sequencing over phenotypic methods include increased reliability and accuracy, rapid identification in 1 to 2 days versus several weeks, providing us with useful phylogenetic information. The wide use and efficacy of 16S rRNA gene sequence analysis is due to its presence in all bacterial species; the gene has a known function and structure with a relatively slow rate of evolution and harbors both conserved and variable regions that allow both genera and species and species-specific motifs to be used for species identification (30). Using nearly full-length 16S rRNA gene sequences for phylogenetic analysis, Ruimy and co-workers in 1994 showed that *Nocardia* species formed a monophyletic clade most closely related to *Gordonia*, *Rhodococcus*, *Mycobacterium*, *Tsukamurella*, and *Corynebacterium*, the most closely related high-G+C genome-containing mycolic acid taxa. Likewise, partial 16S rRNA sequencing by Chun and Goodfellow in 1995 resolved the type strains of 9 *Nocardia* species into a homogeneous taxon distinct from other aerobic actinomycete taxa with the genus Rhodococcus as the closest taxon. Characteristic nucleotide sequences of *Nocardia* were identified and found to be concentrated within helix 37-1 of 16S rRNA [4, 5].

Direct sequencing of 16S rRNA genes also suggested multiple gene copies. Multiple gene copies are usually observed as multiple ambiguous and unresolved overlapping peaks on sequencing chromatographies that cannot be resolved, even after repeated sequencing. The presence of unresolved mixed bases from multiple 16S rRNA gene copies is challenging as they can hamper identification and lead to misidentifications or altered RFLP profiles. Variable numbers of 16S rRNA gene copies may be shared. The presence of heterogeneous gene sequences can complicate identification and phylogenetic analysis of *Nocardia* species isolates. Multiple 16S rRNA gene copies have been detected by cloning and sequencing individual gene copies from single clone isolates or by analysis of whole genome sequences [6].

Conville y colaboradores en 2007 informaron 2, 3 y 5 copias en las cepas tipo *Nocardia de Nocardia concava, Nocardia yamanashiensis y Nocardia ignorata*, respectivamente, después de secuenciar genes clonados de ARNr 16S o estudios de hibridación. El análisis de secuencias del genoma completo de las cepas tipo *Nocardia. Nocardia brasiliensis* HUJEG-1, *Nocardia nova* SH22a y *Nocardia seriolae* UTF1 identificó 3 y 4 copias del gen rRNA, respectivamente. Las bases mixtas se pueden analizar y resolver en algunos casos utilizando el software RipSeq [7].

Conville and coworkers in 2007 reported 2, 3, and 5 copies in the *Nocardia* type strains *Nocardia concava, Nocardia yamanashiensis, and Nocardia ignorata*, respectively, after sequencing cloned 16S rRNA genes or hybridization studies. Whole-genome sequence analysis of the *Nocardia* type strains *Nocardia brasiliensis* HUJEG-1, *Nocardia nova* SH22a, and *Nocardia seriolae* UTF1 identified 3 and 4 copies of the rRNA gene, respectively. Mixed bases can be analyzed and resolved in some cases using RipSeq software [7].

Genetic finger printing by RFLP

Using the PCR technique, it is possible to identify and differentiate between different bacterial strains. The amplification of different genetic elements is a challenge due to the large number of species [8]. Acronym for Restriction Fragments Length Polymorphism. Restriction enzymes are endonucleases that recognize DNA sequences of 4 to 8 base pairs, called restriction targets, and cut at these points. This property can be used to identify polymorphisms, since if one of the nucleotides of the restriction target changes, the enzyme will not be able to cut it. Alternatively, the polymorphism can create a restriction target where there was none. The RFLP technique analyzes the length of the fragments resulting from a digestion with restriction enzymes, allowing the differentiation of alleles according to whether they could be cut or not by the restriction enzyme [9].

The 52 study strains were molecularly identified as Nocardia brasiliensis with similarity percentages greater than 98% with respect to the sequences available in the EzBioCloud database. For the selection of enzymes from the 169 enzymes in the NEBcutter database of New England

BioLabs®, the enzymes *NspI* and *SfcI* were selected for *in silico* restriction. Both showed good restriction patterns, suitable for being organized into ribotypes according to the number of cuts, size of generated bands and distribution [10].

In silico enzyme restriction

In the interphase of the cell cycle, DNA is organized into a double strand of nucleotides that is coiled into a ball. For its study in the laboratory, it is often necessary to fragment it into smaller pieces, which is achieved by means of restriction enzymes (RE). These enzymes, isolated from various bacteria, have the ability to cut the DNA molecule at specific sites, defined for each type of enzyme. There are hundreds of these enzymes, named with acronyms that derive from the bacteria of origin (e.g., *Eco RI, Bam HI, Hind III*). Each enzyme cuts the DNA at a sequence of nucleotides that it recognizes [11].

The cleavage site is called the restriction enzyme site, usually composed of a sequence of 4 to 8 nucleotides. The resulting DNA fragments are known as restriction fragments. A gene may contain multiple restriction sites for different ERs, and its digestion will produce several fragments. The set of these restriction sites in a gene is called a restriction map [11].

From a technical point of view, to cut a specific gene with a restriction enzyme, the DNA and the enzyme are incubated at a suitable temperature for several hours (typically between 3 and 4 hours). The resulting fragments can be separated according to their size by agarose or acrylamide gel electrophoresis. This technique is very useful in the detection of mutations and is used in methods known as RFLP (Restriction Fragment Length Polymorphism) [11].

The restriction patterns generated from the 54 sequences analyzed were classified into 7 clusters or ribotypes. All sequences showed cleavages between 5 and 6 bands, with lengths of 57 bp and 469 bp. Ribotype 1 integrated 31 sequences, the largest cluster, corresponding to 57.4% of the total, among which were the two type strains, ATCC 19296 and DSM 43758. The distribution of 16S rRNA gene sequences according to the *in silico* restriction patterns generated with the enzymes NspI and SfcI [11].

Table 1: Genetics Finger Printig for *Nocardia brasiliensis*

Ribotype	N° of strains per Ribotype	Long of the ARN 16S (pb)	Identification in EzBioCloud	Similarity %	N° of the Fragments	Restriction Fragments Long (pb)					
1	31	1364	*Nocardia brasiliensis NBCR 14402*	99.2	6	465	272	235	223	11	58
2	4	1351	*Nocardia brasiliensis NBCR 14402*	99.75	5	464	272	222	224	111	58
3	2	1342	*Nocardia brasiliensis NBCR 14402*	99.64	6	464	272	223	214	111	58
4	3	1308	*Nocardia brasiliensis NBCR 14402*	99.82	6	464	273	223	179	111	58
5	4	1362	*Nocardia brasiliensis NBCR 14402*	98.83	5	465	456	272	111	58	
6	4	1367	*Nocardia brasiliensis NBCR 14402*	98.62	5	465	282	272	236	112	
7	6	1369	*Nocardia brasiliensis NBCR 14402*	98.8	5	464	388	236	223	58	

Note: Cruz-Medrano M [12]

Identification by MALDI-TOF MS

The *Nocardia* species group could be included among the bacteria that are difficult to identify with the Maldi-Biotyper, one of the main reasons is the database included in the MaldiBiotyper (Bruker, Bremen, Germany), which has few species of those already identified and in turn has few spectra of each species. Thus, the inclusion of new spectra has caused confusion of certain species among themselves, some of which are among the most numerous isolated in our environment, which leads to confusion in the identification of those laboratories that only use MALDI-TOF MS (These are the initials in English for Matrix-Assisted Laser Desorption/Ionization, which means "matrix-assisted laser desorption/ionization", MS being mass spectroscopy for its English acronyms Mass Spectroscopy) as an identification route and do not use molecular biology techniques. Even so, those laboratories that use molecular biology techniques must take into account the genes used and the size of the gene fragment used, as is the case of the 16S rRNA gene considered as the reference gene for typing the *Nocardia* species group [13, 14].

The results between amplification and subsequent sequencing can vary depending on whether the 531bp or 1441bp fragment is used; the latter is the gene size recommended by international organizations such as the CDC. When working with *Nocardia* in the daily laboratory routine for its identification by MALDI-TOF MS, it is done directly from the colony and, failing that, by adding a microliter of formic acid, and depending on the species in question, an optimal identification can be obtained. However, this is not usually the case. Occasionally, the protocol offered by the manufacturer has been tested with formic acid and acetonitrile, but despite this, no improvement in identifications was obtained [13, 14].

In conclusion the 16s rRNA gene is useful for molecular diagnosis of Nocardia brasiliensis. Enzymatic restriction of gene fragments was an effective, rapid and affordable technique to identify Nocardia brasiliensis in a basic molecular biology laboratory. PCR development using PAS-stained polyacrylamide gel is an affordable and rapid method to view the resulting PCCS fragments corresponding to Nocardia brasiliensis

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