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Genetic variability of the 16S rRNA gene of *Nocardia brasiliensis*, the most common causative agent of actinomycetoma in Latin America and the Caribbean

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ABSTRACT

Mycetoma is a neglected tropical disease (NTD) declared by the World Health Organization (WHO) in 2016. It is characterized by the progressive growth of nodules and granulomatous lesions on the legs, arms, and trunk. It is potentially disfiguring and causes disability or amputations in working-age people from marginalized areas. The causative agents can be fungi (eumycetoma) or actinobacteria (actinomycetoma), the latter being the most common in America and Asia. *Nocardia brasiliensis* is the most important causal agent of actinomycetoma in the Americas. Taxonomic problems have been reported when identifying this species, so this study aimed to detect the 16S rRNA gene variations in *N. brasiliensis* strains using an *in silico* enzymatic restriction technique. The study included strains from clinical cases of actinomycetoma in Mexico, isolated from humans and previously identified as *N. brasiliensis* by traditional methods. The strains were characterized microscopically and macroscopically, then subjected to DNA extraction and amplification of the 16S rRNA gene by PCR. The amplification products were sequenced, and consensus sequences were constructed and used for genetic identification and *in silico* restriction enzyme analysis with the New England BioLabs® NEBcutter program. All study strains were molecularly identified as *N. brasiliensis*; however, *in silico* restriction analysis detected a diversity in the restriction patterns that were finally grouped and subclassified into 7 ribotypes. This finding confirms the existence of subgroups within *N. brasiliensis*. The results support the need to consider *N. brasiliensis* as a complex species.

KEYWORDS: Actinomycetoma. Neglected tropical disease. *Nocardia brasiliensis*. *In silico* enzymatic restriction. Genetic variability.

INTRODUCTION

Mycetoma is an anatomoclinical syndrome that has been classified by the World Health Organization (WHO) as a neglected tropical disease (NTD) as of 2016[1]. It is characterized by subcutaneous granulomatous lesions of progressive and painless growth, with the presence of fistulas that drain a serohematic fluid and grains from which the causative biological agent can be isolated. They can produce nodules and abscesses, as well as a deformity in the affected areas, which, depending on their extension, can cause immobility, disability, and amputation of the affected limb. It has local dissemination, in some cases via the lymphatic route, which allows extension into contiguous or distant body regions[2]. The most common presentation is in the lower limbs (78.6%), with the upper limbs (4.0%) and the trunk (6.1%)

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presenting smaller percentage[3]. It is usually diagnosed when the lesions present a significant extension in the affected region, which is sometimes irreversible[4]. Mycetoma can be caused by fungi, called eumycetoma, or actinobacteria, called actinomycetoma[5].

The distribution of mycetoma is traditionally associated with the so-called ‘mycetoma belt’, located around the Tropic of Cancer. The affected countries have tropical or subtropical territories such as Sudan, Nigeria, Senegal, India, Venezuela, Brazil and Mexico[6]. A review study, which included 19,494 cases of mycetoma reported in articles from 1876 to 2019, revealed that the disease has been found in 102 countries, with Sudan (10,608 cases), Mexico (4,155 cases), and India (1,116 cases) being the most affected countries[7]. Actinomycetoma predominates in the Americas and the Middle and Far East[3,7]. The regions where the pathology occurs are mainly rural, of low socioeconomic stratum, with scarce essential housing services and little access to health services, which hinders early detection and treatment while favoring the appearance of complications[3,8,9]. In Latin America and the Caribbean, the most common agent for eumycetoma is *Trematosphaeria grisea*, while for actinomycetoma, it is *Nocardia brasiliensis*[5,7]. The most aggressive presentation of the disease occurs in actinomycetoma[4].

The pathogen enters through the skin, lesions, or open wounds. Farmers are especially susceptible since they do not have protective equipment at work, such as gloves or closed footwear. Patients often seek medical help when the disease has progressed, i.e., when the nodules start to grow or when they cause disability[2,6,8]. Treatment of actinomycetoma requires various antibiotic regimens, including sulfonamides, amikacin, trimethoprim-sulfamethoxazole, and amoxicillin. Treatments may require surgical intervention, such as amputation in the most severe cases[2,4,6].

Although it is a disease with clinical and epidemiological relevance, it is still poorly studied. The latest epidemiological reports of actinomycetoma in Latin America and the Caribbean were carried out in Mexico in 2013[10] and 2014[11], and in Brazil in 2017[12]; all reports came from mycological control centers, hospitals, and healthcare institutions. There are no mycetoma detection and control programs in Latin America[1]. Therefore, the actual prevalence and incidence figures are unknown. In 2021, a bibliometric analysis estimated that, in the last 23 years worldwide, only about 20 articles per year have been published about this disease[13]. As a result, studies on the causal agent of actinomycetoma are also scarce.

From 2012 to the present date, there are only 52 publications on *N. brasiliensis*. Of these, only four have studied its genetic variability and susceptibility to antibiotics[14-17]. With so little information, it is still considered a homogeneous species, which could cause inadequate epidemiological reporting and complicate the clinical approach. This also fails to consider the responses to treatment depending on its possible genetic variations; taking this into account, the study aimed to analyze, from a current perspective, the genetic variability of *N. brasiliensis* isolated from cases of actinomycetoma in Mexico.

MATERIALS AND METHODS

Study strains

The study included 52 strains from samples of clinical cases of actinomycetoma, previously identified as *N. brasiliensis* by traditional methods. The isolation sites of the lesions were the foot, arm, trunk, lung, head and neck. The clinical cases were held in healthcare institutions in Mexico. In addition, two type strains of the same species were included as controls, ATCC 19296 (NR_041860) and DSM 43758 (NR_115828), for a total of 54 study strains.

Culture and growth

The strains, previously preserved in cryotubes at -20 °C, were reactivated in Sabouraud dextrose agar culture medium (BD Bioxon 210700, Mexico, Mexico) with the addition of 1% dehydrated potato and incubated for growth at 37 °C for 21 days. Characterization was performed by macroscopic and microscopic inspection with Gram staining for fragmented mycelia and Gram-positive branched filamentous cells.

DNA extraction

Biomass was obtained by inoculating the strains in Sabouraud dextrose liquid medium (BD Bioxon 222400, Mexico, Mexico) and incubating them in agitation at 37 °C for 30 days. Subsequently, the culture was placed in sterile conical tubes and centrifuged at 6000 X g for 5 min (Labnet, Spectrafuge 16M, Edison, NJ, USA); the biomass obtained was placed in 1.5 ml Eppendorf tubes with sterile saline solution and centrifuged at 8,000 X g for 5 min. The pellet obtained was used for the DNA extraction process.

DNA extraction was performed with the Promega Wizard® Genomic kit (Promega A1125, Madison, USA). The presence and quality of DNA were verified by 1% agarose gel electrophoresis (Bioline BIO-41026, Meridian, Cincinnati, Ohio, USA) in the following conditions:

120 V, 300 µA, 30 min. Ethidium bromide was used as an intercalating agent (Sigma-Aldrich® E7637, Merck, Darmstadt, Germany). DNA purity and concentration were determined by UV spectrophotometry (EPOCH, Agilent, Santa Clara, USA).

16S rRNA gene amplification

Amplification of the 16S rRNA gene was performed by Polymerase Chain Reaction (PCR) in an Axygen® MaxyGene II Thermal Cycler (Axygen, Corning, Arizona, USA) using Taq DNA polymerase (Bioline, BIO-21105, Meridian, Cincinnati, Ohio, USA). The following universal primers were used: 27F: 5'-AGA GTT TGA TCM TGG CTC AG-3' and 1492R: 5'-TAC GGT TAC CTT GTT GTT ACG ACT T-3'. The thermal cycling conditions were: one denaturation cycle of 5 min (94 °C); 30 denaturation cycles for 60 s (94 °C), annealing for 30 s (59 °C), elongation for 60 s (72 °C); finally, one elongation cycle of 10 min (72 °C). The amplified products were visualized in a 1% agarose gel under the previously clear conditions.

Purification and sequencing of PCR product

PCR product purification was performed using the 'PCR Clean up System Promega' kit (Promega A9282, Madison, USA), following the supplier's instructions. The purity and concentration of the amplicon were checked by UV spectrophotometry (EPOCH, Agilent, Santa Clara, USA). The amplified and purified products were sent to the sequencing service of Psomagen (Maryland, USA).

Taxonomic assignment

The sequences obtained were reviewed and corrected using ChromasPro version 2.6.4 (Technelysium, South Brisbane, Queensland, Australia). Consensus sequences were constructed using BioEdit version 7.0.9.0 (Nucleics, Woollahra, Sydney, Australia) and compared with sequences deposited in the EzBiocloud database[18] to determine their percentage similarity.

In silico enzyme restriction

Consensus sequences were subjected to *in silico* enzymatic restriction with the New England BioLabs® NEBcutter virtual tool[19]. Restriction patterns were obtained with the commercial enzymes available in the program's database, with 169 enzymes showing cuts in the study sequences. Subsequently, the enzymes showing the best restriction patterns according to the number and size of the bands were chosen; these parameters were considered to establish the ribotypes.

In silico electrophoresis

With the virtual electrophoresis tool of the New England BioLabs® NEBcutter program, banding models were made simulating a 2% agarose gel and a 100 bp molecular marker, thus obtaining the restriction patterns of the evaluated strains.

RESULTS

Macroscopic and microscopic description

The reactivated strains were inspected macroscopically, according to the characteristics of their colonies, and microscopically with Gram stain. Macroscopically, colonies with irregular and acuminate growth, rough and dry texture, and the presence of aerial mycelium were observed. Differences between strains were also found, such as coloration, in which different shades of orange were notorious, and the appearance of growth, where the relief presented different depths and patterns. Microscopically, branched Gram-positive cells and fragmented mycelia were found in all cases.

Molecular identification

The 52 study strains were molecularly identified as *N. brasiliensis* with similarity percentages greater than 98% compared to the sequences available in the EzBioCloud database. The results are summarized in Table 1.

Restriction enzyme selection

Of the 169 enzymes in the New England BioLabs® NEBcutter database, the *NspI* and *SfcI* enzymes were selected for *in silico* restriction. Both showed good restriction patterns, suitable to be organized into ribotypes according to the number of cuts, size of bands generated, and distribution.

In silico enzyme restriction

The restriction patterns generated from the 54 sequences analyzed were classified into 7 clusters or ribotypes. All sequences showed cuts between 5 to 6 bands, with 57 bp and 469 bp lengths. Ribotype 1 integrated 31 sequences, the most extensive grouping, corresponding to 57.4% of the total, among which were the two type strains, ATCC 19296 and DSM 43758, used as controls in this study. The

Table 1 - Ribotypes, restriction patterns, and molecular identification of 16S rRNA genes of 54 *N. brasiliensis* strains, obtained with *NspI* and *SfcI* enzymes *in silico.*

Ribotype	Number of strains per ribotype	Length 16S rRNA (bp)	EzBioCloud identification	Similarity %	Restriction fragments						
					Number of fragments	Fragment length (bp)					
1	31	1,364	*Nocardia brasiliensis* NBRC 14402	99.20	6	465	272	235	223	111	58
2	4	1,351	*Nocardia brasiliensis* NBRC 14402	99.75	5	464	272	222, 224	111	58	
3	2	1,342	*Nocardia brasiliensis* NBRC 14402	99.64	6	464	272	223	214	111	58
4	3	1,308	*Nocardia brasiliensis* NBRC 14402	99.82	6	464	273	223	179	111	58
5	4	1,362	*Nocardia brasiliensis* NBRC 14402	98.83	5	465	456	272	111	58	
6	4	1,367	*Nocardia brasiliensis* NBRC 14402	98.62	5	465	282	272	236	112	
7	6	1,369	*Nocardia brasiliensis* NBRC 14402	98.80	5	464	388	236	223	58	

distribution of the 54 16S rRNA gene sequences according to the *in silico* restriction patterns generated with *NspI* and *SfcI* enzymes is shown in Table 1.

Virtual electrophoresis

Images of the restriction patterns of each sequence analyzed were obtained by *in silico* electrophoresis. The electrophoreses of each group and their comparison with the other groupings are shown in Figures 1 and 2.

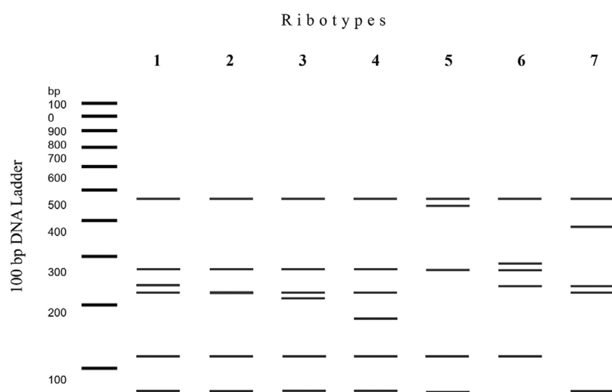

Figure 1 - Virtual electrophoresis model comparison of 7 ribotypes of 16S rRNA gene obtained with *NspI* and *SfcI* enzymes *in silico*, with *N. brasiliensis* strains.

DISCUSSION

Mycetoma is a neglected tropical disease, highly disabling and predominantly observed in rural regions with poor health and housing services. Although its clinical manifestations are notable with the growth of nodules in visible regions of the body such as feet, legs, and arms, it is not usually treated until it presents a considerable extension, deformity, and disability that can lead to amputation[2,4,6,8,9]. Despite being a significant health problem that affects at-risk populations, few studies have addressed it, and today, it is still necessary to discuss the epidemiology, clinical evidence, and etiologic agents of mycetoma.

N. brasiliensis is the most important causative agent of actinomycetoma in Latin America and the Caribbean region[7]. It is a Gram-positive actinomycete, acid-alcohol-resistant, with the formation of intertwined filaments and aerial mycelium[2,20]. It was first mentioned in 1909[21] with the name *Discomyces brasiliensis*. Although it was first described phenotypically and biochemically in 1959[20], its current name was only included in the 'Approved Lists of Bacterial Names' in 1980[22,23]. However, its peculiarities and taxonomic problems have not been solved, especially the detection of variations that indicate a poorly studied diversity within the same species[14-17].

In this study, taking as a sample a collection of 52 strains isolated from clinical cases of actinomycetoma and previously identified by traditional methods, their identity as *N. brasiliensis* was confirmed by sequencing the 16S rRNA gene; however, when approaching a technique capable of analyzing the polymorphic fragments of the gene, the results obtained differentiate them from each other.

The distribution of the study sequences into 7 well-defined and distinct groups demonstrates the variability within the 16S rRNA gene. A significant group is represented by the two type strains and composed of 55.4% of the total sequences. The rest of the sequences form clusters with different restriction patterns showing

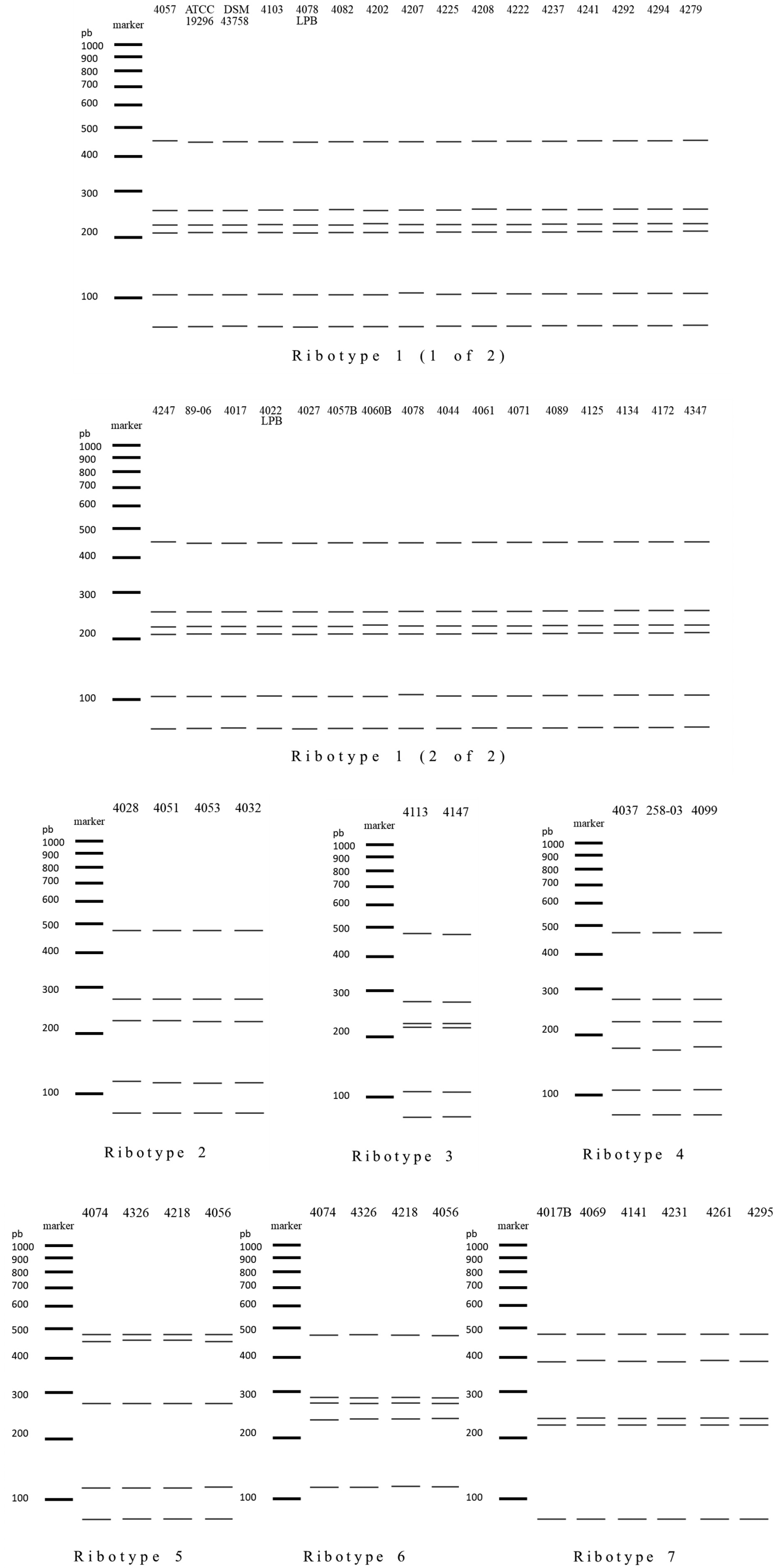

Figure 2 - Virtual electrophoresis model of *in silico* digestion fragments of 16S rRNA genes of 54 *N. brasiliensis* strains, obtained with *NspI* and *SfcI* enzymes.

the discrepancy between strains of the same study species, thus conforming 6 more ribotypes. The results suggest the existence of subgroups within *N. brasiliensis* with more significant variability than previously reported[14-17].

The results coincide and reaffirm those presented in other studies using different methods. Variations within antibiotic susceptibility profiles have previously been detected. In 2014, Schlaberg *et al*.[15] reported significant differences within ceftriaxone susceptibility profiles of 148 strains of *N. brasiliensis* obtained from clinical cases, where the rate of resistance was 51%, demonstrating the intraspecies variability by this method. Coinciding with this, in 2021, Toyokawa *et al.*[17] reported an antibiotic susceptibility study that 14 clinical strains of *N. brasiliensis* showed 57% resistance to ceftriaxone, 21% susceptibility, and 21% intermediate response.

Also, genetic variations have been detected within *N. brasiliensis*. The article published by Chen *et al*.[14] in 2013 performed genotyping of the *gyrB* gene of 12 strains of *N. brasiliensis* obtained from clinical cases, reporting variations that distributed the strains into two large groups that, in addition, showed differences in terms of clinical characteristics, such as the presence of lymphadenitis and susceptibility to imipenem.

In the study published by Kosova-Maali *et al*.[16] in 2018, where they worked with 36 strains of *N. brasiliensis* obtained from clinical cases, they were able to identify three well-defined genotypes by polymorphisms of the constitutive genes *hsp65* and *sodA*; this finding related the genotype and clinical presentation of actinomycetoma among the different strains. In addition, the similarity percentages with reference strains were reported to range from 99.39% to 99.57%.

To broaden and deepen the genetic variability found, highly discriminatory studies such as enzyme restriction could be performed on other constitutive genes in *N. brasiliensis*. *In silico* enzyme restriction studies are useful for expanding the number of enzymes available in the assay and thus making an appropriate choice of enzymes[24-26], but they are also useful for reducing costs and materials in the experiment because they have a high coincidence with the results shown *in vitro*[26-29].

CONCLUSION

Previously, *in silico* enzymatic restriction studies have demonstrated the intraspecies genetic variability in other bacteria, such as identifying clusters and subclusters in the streptokinase gene of *Streptococcus*[26], the identification of *E. coli* genotypes, which also suggested updating the existing classifications[30]. Its usefulness was also proven in constructing new classifications in the study species, with a highly reliable discrimination power, such as the one performed using the *cpn60* gene of phytoplasmas[28]. Employing enzymatic restriction of the 16S rRNA gene, variations of pathogenic bacteria, such as *P. salmonis*, have also been described, detecting differences between the LF-89 and EM-90 genotypes[25]. Thus, this study is the first to address the genetic variability of *N. brasiliensis* through the *in silico* enzymatic restriction technique in the 16S rRNA gene.

Exposing and delving into the existence of diversity within the same species with medical and epidemiological relevance, such as *N. brasiliensis*, helps to understand part of the taxonomic complexity it presents and contribute to its knowledge and epidemiological report in the future.

With the previous reports and the results obtained in this study, it is supported that *N. brasiliensis* is a complex of species. In this study, seven possible groups within the complex have been established. Establishing this actinomycete as a species complex is essential for its taxonomic understanding and properly informing its epidemiology.

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