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Nontuberculous mycobacteria from mexican archaeological sites

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Abstract

We examined several buildings of nine archaeological sites in Mexico for the presence of mycobacteria and we could isolate forty-five nontuberculous mycobacteria (NTM). These were isolated from biofilms using selective media containing different antibiotics and dyes. Identification of the isolated mycobacteria was carried out, first, by a molecular identification by means of a mycobacteria-specific PCR using bacterial lysates of the acid-fast bacilli followed by species identification by comparing of three molecular markers: genes *rrs* (*16SrRNA*), *hsp*65 and *rpo*B. Furthermore, the physiographic data of the archaeological zones under study was related to the number of acid-fast microorganisms using a univariate analysis of variance. From the 45 isolated mycobacteria, 21 were *Mycobacteroides chelonae*; seven, *Mycobacteroides abscessus*; five, *Mycolicibacterium flavescens*; four, *Mycobacterium alvei*; two, *Mycobacterium fortuitum*; and six, *Mycobacterium* sp. Most NTM were isolated from two archaeological sites: 25 from Guachimontones (Jalisco), and 13 from Atetelco (Estado de México). The statistical analysis showed that environmental factors such as climate and the temperature-humidity-precipitation interaction had the greatest influence on the presence of NTM in these archaeological zones.
Key Words: environmental nontuberculous mycobacteria, biofilms, archaeological sites.

Micobacterias no tuberculosas de sitios arqueológicos de México

Resumen

Se examinaron varios edificios de nueve sitios arqueológicos en México para detectar la presencia de micobacterias y se pudieron aislar 45 cepas no tuberculosas. Se obtuvieron a partir de biopelículas usando medios selectivos con antibióticos y colorantes. Las cepas aisladas se determinaron como micobacterias mediante una PCR específica, posteriormente se identificaron con tres marcadores moleculares: genes *rrs* (*16S rRNA*), *hsp*65 y *rpo*B. Además se relacionaron los datos fisiográficos de las zonas arqueológicas estudiadas con el número de bacterias ácido alcohol resistentes mediante un análisis univariado de varianza. De las 45 cepas de micobacterias aisladas 21 correspondieron a *Mycobacteroides chelonae*; siete, *M. abscessus*; cinco, *Mycolicibacterium flavescens*; cuatro, *Mycobacterium alvei*; dos, *M. fortuitum*; y seis quedaron como *Mycobacterium* sp. La mayoría de las micobacterias no tuberculosas (MNT) fueron aisladas de dos de las zonas arqueológicas: 25 de Guachimontones (Jalisco) y 13 de Atetelco (Estado de México). El análisis estadístico mostró que los factores ambientales como clima y la interacción temperatura-humedad-precipitación tuvieron una gran influencia en la presencia de este grupo bacteriano en las zonas arqueológicas.
Palabras Clave: micobacterias no tuberculosas ambientales, biopelículas, zonas arqueológicas.

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Introduction

Archaeological remains are limited and non-renewable resources of valuable information about ancient civilizations; consequently, they may potentially contribute to a deeper understanding of human societies in previous times. Archaeological sites are important not only for education and research purposes, but also from tourism, cultural and recreational perspectives. Both archaeological monuments and historical buildings are exposed to multiple environmental factors that make them vulnerable to deterioration and destruction, such as humidity, high temperatures, precipitation, anthropogenic activity and the effect of micro- and macro-biological communities (Videla, Guiamet & Gómez de Saravia, 2000).

Biodeterioration is a term that collectively refers to the damages caused by microbiological communities in archaeological sites and historic buildings as a consequence of the metabolism of endo- and epilithic microorganisms that degrade the rocky substrates where they thrive as a result of the production of various metabolites such as organic and inorganic acids (Ascaso, Wierzchos, Souza-Egipsy, De los Ríos & Delgado Rodrigues, 2002).

Microbial communities frequently produce biofilms, i.e., structured communities that include bacteria, algae, cyanobacteria, fungi and protozoa, embedded in a polymer matrix that provides support and protection and serves as a nutrient reservoir (Romaní, Found, Artiagas, Schwartz, Sabater & Obst, 2008**)**. Microorganisms that are capable to colonize and survive in inhospitable environments play a key role in the establishment of microbial populations that contribute to the formation of soils from the substrates on which these grow (McNamara, Perry, Bearce, Hernández-Duque & Mitchell, 2006).

Recently, the family Mycobacteraceae has been subdivided into five different genera: *Mycobacterium*, *Mycolicibacter*, *Mycobacteroides*, *Mycolicibacillus*, and *Mycolicibacterium* (Gupta, Lo & Son, 2018). These mycobacteria are short aerobic bacilli, straight or slightly curved, and non-motile. The high G+C content coupled with a cell envelope rich in mycolic acids and other lipids make them resistant to discoloration by acid-alcohol when stained with the Ziehl-Neelsen technique. According to their *16S rRNA* sequences and the disease they can cause, mycobacteria have been classified in two groups: the *Mycobacterium tuberculosis* complex (MTC) and nontuberculous mycobacteria (NTM). Some mycobacteria cause infections in humans and/or animals, while others are colonizing organisms that form biofilms. Studies on these organisms have focused primarily on the clinical and veterinary areas; consequently, the search for them has been done on soil and drinking water samples, as well as in man and other mammals (Falkinham, 2009a; Johnson & Odell, 2014).

Nontuberculous mycobacteria are ubiquitous microorganisms that inhabit terrestrial and aquatic environments and have been isolated from various sources, some of which are directly linked to human environments (Falkinham, 2015), as shown by the report of the isolation of five strains of mycobacteria that might be involved in the deterioration of monuments in Angkor, Cambodia from 2011; according to the author, all five strains isolated were able to use elemental sulfur (S^0) for chemolithoautotrophic growth, and organic substances for chemoorganoheterotrophic growth (Kusumi, Shu-Li & Katayama, 2011). To the best of our knowledge, the latter is the first report of NTM isolated from such environments; therefore, the aim of the present work was to search, isolate and identify NTM from biofilms grown on stone monuments of various archaeological sites in Mexico.

Materials and methods

Sample Collection

Biofilm samples were collected aseptically using a sterilized spatula and placed in a sterile polypropylene container with screw cap. Samples were collected mainly in the rainy season (July, August, and September), from deteriorated buildings located in nine Mexican archaeological sites (Figure 1), namely Guachimontones (Jalisco), Atetelco and Malinalco (Estado de México), Zaachila (Oaxaca), Tulum (Quintana Roo), EkBalam (Yucatán), Tizatlán and Xochitécatl (Tlaxcala), and La Antigua (Veracruz). Sampling was conducted with proper care to avoid damaging the monuments (non-invasive method). During the sampling, the most deteriorated points of monuments were chosen, where well-consolidated biofilms or a light layer of soil (not thicker than 2 cm) were observed; the characteristics of all collection sites were recorded (Table I).

Microorganism Isolation

Acid-fast microorganisms were isolated by suspending 2 g of sample in 20 mL sterile distilled water and grinding with 710-1180 µm glass beads (Sigma™) in Vortex™ set at maximum speed; afterwards, these were left to stand at room temperature for 1 h. The supernatant suspension (~18 mL) was mixed with an equivalent volume of 0.75% (w/v) hexadecylpiridinium chloride (HPC) by inversion, and was left to settle for 18 h at room temperature. Afterwards, the suspension was centrifuged (5,000 xg at 25 ºC for 20 min) and decanted. The pellet was suspended in 1 mL of sterile water and distributed and grown on two Lowenstein-Jensen (LJ) medium slopes (Thorel, Falkinham & Moreau, 2004); one LJ slope was incubated at 28 ºC and the other at 37 ºC, both for 8 weeks. Once a typical mycobacterial growth was observed, each colony was stained with the Ziehl-Neelsen technique; colonies containing acid-fast bacilli (AFB) were subcultured by the

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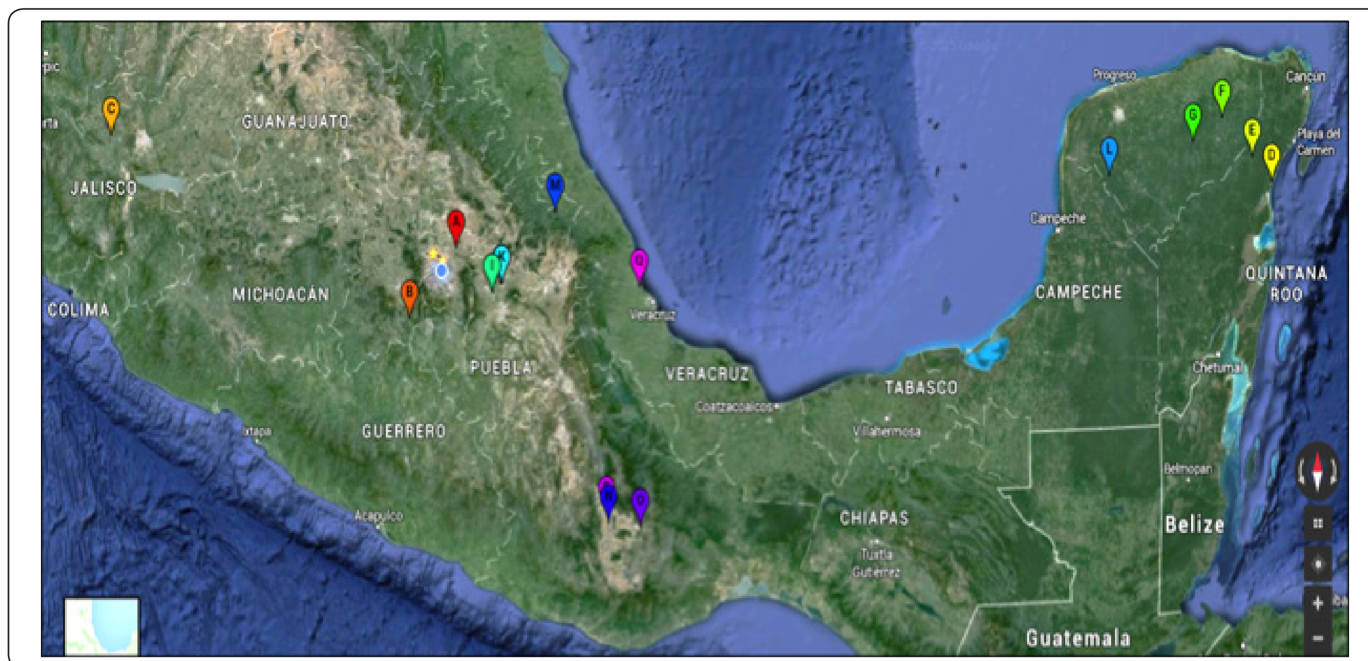

Figure 1. Location of archaeological sites under study. A: Atetelco, Estado de México; B: Malinalco, Estado de México; C: Guachimontones, Jalisco; D: Tulúm, Quintana Roo; E: Cobá, Quintana Roo; F: EkBalam, Yucatán; G: ChichénItzá, Yucatán; H: Cacaxtla, Tlaxcala; I: Xochitécatl, Tlaxcala; J: Ocotelulco, Tlaxcala; K: Tizatlán, Tlaxcala: L: Uxmal, Yucatán; M: Yohualichan, Puebla; N: Monte Albán y Zaachila, Oaxaca; O: Mitla, Oaxaca y Q: La Antigua, Veracruz. Figure designed by the authors.

streak-plate method on Middlebrook 7H10 agar enriched with 10% albumin-dextrose-catalase (ADC) and 0.5% glycerol. Some cases required sub culturing by streak-plate method in enriched Middlebrook 7H10 agar with added malachite green (0.4g/L) or PANTA^{TM} antibiotic mixture (polymyxin B 30,000 µg/L, amphotericin B 3,000 µg/L, nalidixic acid 12,000 µg/L, trimethoprim 3,000 µg/L, and azlocylin 3,000 µg/L). In all cases, incubation temperature was the one at which colonies originally grew. Isolates obtained in enriched Middlebrook 7H10 agar were described in terms of colony and microscopic morphologies; after incubation in Dubos broth enriched with 10% ADC, isolates were preserved in 2 mL cryotubes at -70 °C with 15% glycerol.

Isolates Identification

Isolates were identified using bacterial lysates obtained by suspending one loopful of growth in 250 µL sterile water and subjected to three cooling/heating cycles (5 min at 4 ºC; 5 min at 100 ºC). The molecular identification of the family Mycobacteriaceae was established through a specific PCR assay for the amplification of a 900-1,500 bp fragment covering from the last 99 codons of the *mur*A gene to the position 357 of the *rrs* gene for the different genera using standard Taq DNA polymerase (Life Technologies, Rockville, MD), and primers RAC1 (5'-TCGATGGTCACCGAGAACGTGTTC-3') and RAC8 (5'-CACTGGTGCCTCCCGTAGG-3'), to make a total reaction volume of 50 µL (González-y-Merchand, Colston & Cox, 1996; Cobos-Marín, Rivera-Gutiérrez, Licea-Navarro, González-y-Merchand & Estrada-García, 2003).

The nontuberculous mycobacteria (NTM) isolated were identified by comparing the nucleotide sequence of three genes: *hsp*65, *rrs*and *rpo*B, using: (i) the restriction pattern analysis of a *hsp*65 gene fragment that encodes the 65kDa heat shock protein (PRA) (Telenti *et al*., 1993); (ii) sequencing of the hypervariable 2 region (V2) of the *rrs* gene (*16S rRNA*) (Kirschner, Parker & Falkinham, 1999); and (iii) sequencing of the variable V region of the *rpo*B gene (Adékambi, Colson & Drancourt, 2003). The PRA technique consisted in the amplification of a 439-bp fragment of the *hsp*65 gene using primers TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3') (Telenti *et al*., 1993). This amplicon was digested in two separate reactions with two restriction enzymes, *Bst*EII (New England Biolabs) and *Hae*III (Invitrogen). The products from both digestions were observed in a 3% agarose gel stained with ethidium bromide (83 µg/mL) to obtain the respective restriction patterns. Band sizes were compared against the available database (PRASITE, 2018). The species of mycobacteria were also identified by sequencing the V2 region of the *rrs* gene and the V region of the *rpo*B gene. The V2 region of the *rrs* gene was amplified with primers RAC1/RAC8; the V region of the *rpo*B gene was amplified with primers MycoF (5'-GGCAAGGTCACCCCGAAGGG-3') and MycoR

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Archaeological sites	State	Altitude (msnm)	Climate	Humidity	Mean annual precipitation (mm)	Mean annual temperature (°C)	Construction materials
Atetelco (Teotihuacán)	Estado de México	2,270	Semi-dry with summer rainfall	Low	600 - 700	14 - 16	"Tepetate" or volcanic tolba with and without caliche (calcium carbonate)
Malinalco		1,750	Semi-warm subhumid with summer rainfall	High	800 - 1,300	14 - 28	Extrusive igneous rock, and limestone
Guachimontones	Jalisco	1,260		Medium	800 - 1,100	16 - 22	Stone, adobe, and lime
Tulúm	Quintana Roo	5	Warm subhumid with summer rainfall	High	0 - 1,300	24 - 28	Limestone
Cobá		15					
Ek Balam	Yucatán	22		Medium	1,000 - 1,200		
Chichén Itzá		20			1,100 - 1,200		
Uxmal		31			1,000 - 1,100	24 - 28	
Cacaxtla	Tlaxcala	2,200	Temperate subhumid with summer rainfall		800 - 1,000	14 - 16	"Tepetate", limestone, adesitas, "tezontle", and adobe.
Xochitécatl							
Ocotelulco		2,340					Stone slabs of xalnene, adobe, stone, tepetate, and limestone.
Tizatlán		2,240					"Tezontle"and limestone
Mitla	Oaxaca	1,680	Temperate subhumid with summer rainfall		600 - 1,500	14 - 22	Limestone
Monte Albán		1,580	Semi-dry semi-warm	Low	600 - 700	18 - 22	Stone, adobe, and stucco
Zaachila		1,490			600 - 800	16 - 22	Limestone
La Antigua	Veracruz	20	Warm subhumid with summer rainfall	High	1,100 - 1,300	24 - 26	Coral, river stone, brick quarry, flat tile, and stucco
Yohualichan	Puebla	600	Semi-warm wet with rain all year		1,900 - 4,100	18 - 26	Limestone

Table I. Characteristics of the archaeological zones studied.

(5'-AGCGGCTGCTGGGTGATCATC-3') to obtain a 723-bp fragment (Adékambi, Colson & Drancourt, 2003). Both PCR products were sequenced using the primers RAC8 and MycoF, respectively, with the Big Dye terminator ready-reaction kit (Perkin-Elmer, Inc., Wellesley, MA); sequences were analyzed with the ABI PRISM 310 Genetic Analyzer system (Perkin-Elmer). The bioinformatic analysis of the sequences obtained consisted of an alignment with known sequences of the NCBI GenBank database in search of similarities (Identity ≥ 97%) using the BLASTn program.

Statistical Analysis

The relationship between physiographic data of the areas studied and the number of AFB microorganisms found was explored through a univariate analysis of variance (α= 0.2) using the general linear model and the IBM-SPSS V program

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22; this analysis evaluated the different physiographic factors of the studied areas (altitude, climate, mean annual precipitation, humidity, mean annual temperature, construction material, and precipitation-humidity-temperature interaction).

Results

Fifty acid-fast microorganisms were isolated from the nine archaeological sites. Table II shows the distribution of these organisms; the largest number of isolates was obtained from Guachimontones and Atetelco. In contrast, the sites with the lowest number of isolates were Tizatlán, La Antigua, Tulum, and EkBalam. The remaining isolates were distributed among Xochitécatl, Zaachila and Malinalco.

Site	State	Number of isolates	Species
Tizatlán	Tlaxcala	1	*M. chelonae*
Xochitécatl	Tlaxcala	2	*Mycobacterium* sp.
Atetelco	Estado de México	12	*M. alvei*, *M. flavescens*, *M. fortuitum*, *Mycobacterium* sp.
Malinalco	Estado de México	4	*M. chelonae*
Guachimontones	Jalisco	26	*M. abscessus* *M. chelonae* *Mycobacterium* sp.
Zaachila	Oaxaca	2	*M. abscessus*
La Antigua	Veracruz	1	*M. chelonae*
EkBalam	Yucatán	1	*Mycobacterium* sp.
	TOTAL	**49**	

Table II. Acid-fast microorganisms isolated by archaeological site.

The comparisons of environmental factors with the number of AFB microorganisms through the univariate analysis of variance (general linear model) was significant only for climate (sig. = 0.127, Eta = 0.239) and for the precipitation-humidity interaction-temperature (sig. = 0.193; Eta = 0.180); that is, these were the factors with the greatest influence on the presence of AFB microorganisms in archaeological zones.

The morphological descriptions of the isolates obtained were conducted in Middlebrook 7H10 medium after 6 and 15 days of incubation at 28 °C. Colonies were either circular or irregular, measuring 1.5-15 mm in diameter, and white, yellow or light yellow; most were flat or umbonate and soft, with no other distinctive characteristics. All isolates were acid-fast bacilli, measuring 1-3 µm in length. The Mycobacteriaceae-specific PCR assay tested positive for 45 of the 50 AFB isolates, i.e., 90% of isolates were mycobacteria. The results of the three molecular markers (*rrs*, *hsp*65, and *rpo*B genes) serve to define 39 species of the 45 NTM isolated; five consensus species were determined, distributed as follows: 21 isolates were *Mycobacteroides chelonae*; seven, *Mycobacteroides abscessus*; five, *Mycolicibacterium flavescens*; four, *Mycobacterium alvei*; and two, *Mycobacterium fortuitum*; the six that could not be identified to species through the comparison of the three markers used were reported as *Mycobacterium* sp. (Table III). The Gene Bank access numbers are listed on Table IV, these species were isolated from eight of the nine archaeological sites. The largest number of NTM (25) was isolated from Guachimontones (Jalisco), corresponding to at least two different species; the site that ranked second in number of NTM isolated was Atetelco (Estado de México), with at least three different species.

Discussion

We investigated the biofilms that grow on monuments of different archaeological sites of Mexico searching for cultivable mycobacteria; this required performing decontamination and isolation procedures during which mycobacteria were isolated from the microbial consortium where they were immersed. The course of these techniques required microscopic observations to identify mycobacteria based on acid fastness. However, this trait is not unique to mycobacteria, and a number of other acid-fast microorganisms of different shapes, which coexist with NTM in their natural environment, were observed throughout the procedures.

Microorganisms do not live in isolation; they rather form complex communities (such as biofilms) that allow them to with stand the fluctuating environmental conditions. In addition, microbial assemblages display a differential response to climatic variations, so that their composition is constantly changing over time and space (Garret, Bhakoo & Zhang, 2008). A particular case was observed in a sample from Guachimontones, where *Mycobacterium* sp. was found along with a filamentous acid-fast microorganism with which the former was so closely associated that it was necessary to incubate both microorganisms together in liquid medium followed by their subsequent subculture by cross streak method in Middlebrook 7H10 agar. This was not the only close association observed during the isolation process; in general, mycobacteria grew with in a microbiota assemblage that made it difficult to obtain pure cultures, mainly due to the time required to observe growth.

Most mycobacterial isolates developed after 6 days of incubation at 28 °C (fast-growing NTM); however, this generation time is too long relative to the one of other bacteria that, when cultivated together, displayed a faster growth compared to mycobacteria. This issue was solved by adding either antibiotics (PANTA^{TM}) or malachite green (0.4 g/L) to the culture medium.

Key	Results Region V2 (*rrs*)	Results PRA (*Hsp*65)	Results Region V (*rpo*B)	Consensus
38A Xoc	*M. abscessus, M. chelonae, M. massiliense, M. salmoniphilum, M. bolletii, M. immunogenum, M. fortuitum*	*M. massiliense, M. bolletii, M. abscessus*(Type 2)	*M. saopaulense, M. chelonae, M. immunogenum, M. abscessus, M. massiliense*	*Mycobacterium* sp.
38B Xoc	*M. abscessus, M. chelonae, M. massiliense, M. salmoniphilum, M. bolletii, M. immunogenum, M. fortuitum*	*M. massiliense, M. bolletii, M. abscessus*(Type 2)	*M. saopaulense, M. chelonae, M. immunogenum*	*Mycobacterium* sp.
285A Gua	*M. salmoniphilum, M. bolletii, M. massiliense, M. chelonae, M. abscessus*	*M. massiliense M. bolletii M. abscessus*(Type 2)	*M. saopaulense, M. chelonae, M. immunogenum*	*Mycobacterium* sp.
588 Ate	*M. abscessus, M. chelonae, M. massiliense, M. salmoniphilum, M. bolletii, M. immunogenum, M. fortuitum*	*M. massiliense M. bolletii M. abscessus*(Type 2)	*M. bolletii, M. chelonae, M. abscessus M. massiliense*	*Mycobacterium* sp.
590 Gua	*M. abscessus, M. chelonae, M. massiliense, M. salmoniphilum, M. bolletii, M. immunogenum, M. fortuitum*	*M. abscessus*(Type 1*)*	*M. saopaulense, M. immunogenum, M. chelonae*	*Mycobacterium* sp.
600 EkB	*M. abscessus, M. chelonae, M. massiliense, M. salmoniphilum, M. bolletii, M. immunogenum, M. fortuitum*	*M. massiliense, M. bolletii, M. abscessus*(Type 2)	*M. abscessus, M. massiliense, M. bolletii, M. chelonae, M. immunogenum*	*Mycobacterium* sp.

Table III. Results of the three markers for the six NTMs that could not be identified to species.

In nature, both the impermeability of the cell envelope and the time taken for mycobacteria to grow give them an advantage relative to other coexisting organisms. The synthesis of this highly complex envelope made of mycolic acid requires mycobacteria to invest a longer time, thus affecting growth rate. This key adaptation, due to the hydrophobicity it confers, enables mycobacteria to attach to surfaces and remain attached to the substrate even despite water runoff. Furthermore, this impermeable barrier protects them from dehydration in dry seasons, as well as from a number of chemicals under lab conditions, such as antibiotics and dyes (Falkinham, 2009b; Falkinham, 2015).

Most isolates came from sampling points where moss was also present, forming a consolidated biofilm on the stones that make up the foundation of the buildings under study; this is consistent with other investigations that report mycobacteria in association with bryophytes (Pavlik, Kazda & Falkinham, 2010; Thorel, Falkinham & Moreau, 2004). Apparently, the life cycle and seasons of the year when the various species of bryophytes grow are directly related to the establishment and development of mycobacteria, i.e., during the growth of mosses (early spring to late autumn) a green hygroscopic layer develops that serves as habitat for various species of microorganisms. Each year, moss regrows to form a new layer on top of the one of the previous year, leaving the latter deprived from light; as this accumulates over time, the deeper layers are broken down by pectinases from mycorrhizal fungi. The products released from the metabolism of fungi and the decomposition of mosses (amino acids and carbohydrates) are dissolved in the medium, producing a suitable environment for the development of mycobacteria (Pavlik, Kazda & Falkinham, 2010). Mycobacteria were also found on biofilms devoid of moss and with low humidity (Atetelco and Zaachila), a finding that can be explained by the same adaptations developed over the course of the evolutionary history (hydrophobicity, impermeability, and adherence) that allows them to survive under extreme conditions such as desiccation or low nutrient levels.

The 45 colonies identified as NTM showed the typical characteristics of the genus *Mycobacterium*; however, during the isolation process some colonies (later identified as the same species), developed into two different morphologies (usually flat or umbonate), leading to uncertainty about their identity. It has been reported that the chemical structure of

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Key	Gene Bank access number *srrs*	Gene Bank access number *srpoB*	Specie
38A	*KX891468*	*MK603440*	*Mycobacterium* sp.
38B	*KX891469*	*MK603441*	*Mycobacterium* sp.
39A	*KX891471*	*MK603443*	*M. flavescens*
39B	*KX891472*	*MK603444*	*M. flavescens*
39C	*KX891473*	*MK603445*	*M. flavescens*
58A	*KX891480*	*MK603452*	*M. flavescens*
58B	*KX891481*	*MK603453*	*M. flavescens*
62A	*KX891484*	*MK603456*	*M. chelonae*
62B	*KX891485*	*MK603457*	*M. chelonae*
62C	*KX891486*	*MK603458*	*M. chelonae*
62D	*KX891487*	*MK603459*	*M. chelonae*
62E	*KX891488*	*MK603460*	*M. chelonae*
62F	*KX891489*	*MK603461*	*M. chelonae*
65A	*KX891490*	*MK603462*	*M. chelonae*
65B	*KX891491*	*MK603463*	*M. chelonae*
65C	*KX891492*	*MK603464*	*M. chelonae*
66A	*KX891493*	*MK603465*	*M. chelonae*
66B	*KX891494*	*MK603466*	*M. chelonae*
66C	*KX891495*	*MK603467*	*M. chelonae*
66D	*KX891496*	*MK603468*	*M. chelonae*
125	*KX891452*	*MK603424*	*M. abscessus*
126A	*KX891453*	*MK603425*	*M. abscessus*
126B	*KX891454*	*MK603426*	*M. abscessus*
254A	*KX891455*	*MK603427*	*M. chelonae*
254B	*KX891456*	*MK603428*	*M. chelonae*
256	*KX891457*	*MK603429*	*M. chelonae*
285A	*KX891458*	*MK603430*	*Mycobacterium* sp.
286A	*KX891459*	*MK603431*	*M. abscessus*
286B	*KX891460*	*MK603432*	*M. abscessus*
287	*KX891461*	*MK603433*	*M. alvei*
288	*KX891462*	*MK603434*	*M. alvei*
290	*KX891463*	*MK603435*	*M. fortuitum*
292	*KX891464*	*MK603436*	*M. fortuitum*
319A	*KX891465*	*MK603437*	*M. chelonae*
319B	*KX891466*	*MK603438*	*M. chelonae*
380	*KX891467*	*MK603439*	*M. alvei*
391	*KX891470*	*MK603442*	*M. chelonae*
410A	*KX891474*	*MK603446*	*M. chelonae*
466A	*KX891475*	*MK603447*	*M. abscessus*

Table IV. Gene Bank access numbers of the isolated species of mycobacteria.

Key	Gene Bank access number *srrs*	Gene Bank access number *srpoB*	Specie
466B	*KX891476*	*MK603448*	*M. abscessus*
566	*KX891477*	*MK603449*	*M. alvei*
578	*KX891478*	*MK603450*	*M. chelonae*
588	*KX891479*	*MK603451*	*Mycobacterium* sp.
590	*KX891480*	*MK603454*	*Mycobacterium* sp.
600	*KX891483*	*MK603455*	*Mycobacterium* sp.

Table IV. Gene Bank access numbers of the isolated species of mycobacteria (*Cont.*).

the mycobacterial cell envelope changes under stressful conditions (e.g., exposure to antibiotics), by rearranging the peptidoglycan layer or during an infection (bacteria-host interaction), by increasing the production of mycolic acids and other cell-envelope lipids (Yang *et al.*, 2013). Some studies have shown the production of mutants with different morphological characteristics after exposure to antibiotics such as rifampicin and ethambutol or to sanitizing agents such as glutaraldehyde. In these cases, changes in the composition of fatty acids and the arabinogalactan/arabinomannan ratio in the mycobacterial cell envelope have been detected (Manzoor, Lamber, Griffiths, Gill & Fraise, 1999; Sareen & Khuller, 1990). In addition, it has been reported that these microorganisms display a high plasticity in the synthesis of the cell envelope, thus facilitating the development of mutants that are more resistant to adverse environmental conditions (Kieser & Rubin, 2014). Our observations during the isolation of NTM from archaeological sites are consistent with the hypothesis that the differentiation of colony morphology occurs in response to stress, as this phenomenon was observed when the antibiotic mixture was added to the culture medium of some samples or upon exposing them to malachite green. This likely led to the selection of mutant strains of the same species, since colonies did not return to their original morphology upon re-inoculation in medium devoid of antibiotics; alternatively, these colonies may have developed other morphotypes of the same NTM species when the growth of other microorganisms was inhibited. Furthermore, our observations regarding growth in liquid medium revealed that flat morphotypes were more hydrophobic than the umbonated isolates. All strains that differentiated in the same medium shared the same identification at species level (*M. abscessus, M. chelonae*, and *M. flavescens*).

Conclusions

In this pilot study, five different species of NTM were successfully isolated from several archaeological sites in Mexico. Most were part of biofilms grown on stones of

monuments and were associated to other microorganisms, a relationship that deserves further investigation in order to gain a deeper insight on the role of these NTM in such habitats.

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