Article
ECORFAN Journal Republic of Guatemala
June 2018 Vol.4 No.6 12-16

Molecular identification of mycobacteria species present in patients with cutaneous tuberculosis in Yucatán, Mexico

Identificación molecular de especies de micobacterias presentes en pacientes con tuberculosis cutánea en Yucatán, México

CAAMAL-LEY, Ángel D. †*, PUC-FRANCO, Miguel A., ANDUEZA-PECH, María G., CERÓN-ESPOINOSA, José D. and VARGAS-GONZÁLEZ, Alberto

Centro de Investigaciones Regionales Dr. Hideyo Noguchi". Universidad Autónoma de Yucatán

ID 1st Author: Ángel D., Caamal-Ley

ID 1st Coauthor: Miguel A., Puc-Franco

ID 2nd Coauthor: María G., Andueza-Pech

ID 3rd Coauthor: José D., Cerón-Espoinosa

ID 4th Coauthor: Alberto, Vargas-González

Received March 10, 2018; Accepted June 30, 2018

Abstract

The control of tuberculosis is a priority in the policies of the Ministry of Health in Mexico. In the present work, the detection of Mycobacterium sp. Was performed through the Polymerase Chain Reaction (PCR), as well as smear and culture tests. The study was conducted with 8 skin biopsies from different patients with suspected cutaneous tuberculosis, from the Dermatological Center of the State of Yucatan. Bacteriological studies were performed using the Ziehl-Neelsen method and the culture was carried out by incubation of the samples in Lowenstein-Jensen medium at 37 ° C for eight weeks. For PCR, the DNA was amplified with a specific pair of primers for the Hsp65 gene, retaining in all Mycobacterium spp. a size of 439 bp of expected amplification. The growth of Mycobacterium spp. Was observed in three samples after incubation in Lowenstein-Jensen media. The PCR-RFLP indicated other species of Mycobacterium (M. abcessus, M. fortuitum and M. leprae). The sensitivity of the PCR in relation to culture and sputum smear was 87.5%

Tuberculosis, Mycobacterium, Sputum Smear, Culture, PCR

Resumen

El control de la tuberculosis es una prioridad en las políticas de la Secretaría de Salud en México. En el presente trabajo, se realizó la detección de *Mycobacterium sp*., mediante la Reacción en Cadena de la Polimerasa (PCR), así como pruebas de baciloscopía y cultivo. El estudio se realizó con 8 biopsias de la piel de diferentes pacientes con sospecha de tuberculosis cutánea, del Centro Dermatológico del estado de Yucatán. Los estudios bacteriológicos, se realizaron mediante el método de Ziehl-Neelsen y el cultivo se llevó a cabo mediante la incubación de las muestras en medio Lowenstein-Jensen a 37 ° C durante ocho semanas. Para la PCR, el ADN se amplificó con un par específico de cebadores para el gen Hsp65, conservando en todos *Mycobacterium spp*. un tamaño de 439 pb de amplificación esperado. El crecimiento de *Mycobacterium spp*., se observó en tres muestras después de la incubación en medios de Lowenstein-Jensen. El PCR-RFLP indicó otras especies de *Mycobacterium* (*M. abcessus, M. fortuitum y M. leprae*). La sensibilidad de la PCR en relación con el cultivo y la baciloscopia fue del 87,5%.

Tuberculosis, Mycobacterium, Baciloscopia, Cultivo, PCR

Citation: CAAMAL-LEY, Ángel D., PUC-FRANCO, Miguel A., ANDUEZA-PECH, María G., CERÓN-ESPOINOSA, José D. and VARGAS-GONZÁLEZ, Alberto. Molecular identification of mycobacteria species present in patients with cutaneous tuberculosis in Yucatán, Mexico. ECORFAN Journal-Republic of Guatemala. 2018, 4-6: 12-16.

* Correspondence to Author (email: andueza@correo.uady.mx)
† Researcher contributing first author.

Introduction

Mycobacterioses are infections caused by mycobacteria other than those belonging to the Mycobacterium Tuberculosis Complex (MTC). The current situation of these diseases is unknown, because neither infections nor isolates are reported. Even so, it is believed that the prevalence of non-tuberculous mycobacteria (NTM) is increasing every year, possibly due to the fact that human beings are more in contact with certain types of environments, as well as the demographic changes of the population and the population. increase in people with some immunosuppression (Cassidy PM., y cols., 2009; Lee AS., y cols., 2009; Glapinski J., y cols., 2004; Yew WW., y col., 2011)

Most of the pathogenic species of MNT can cause skin and soft tissue infections, many of them are distributed worldwide and are fast growing, among the main ones, we find M. fortuitum, M. chelonae, M. abscessus, M. marinum and M. ulcerans (Amresh Kumar Singh y cols. 2015). In the state of Yucatán, the diagnosis is made through skin biopsies of patients with symptoms suggestive of this disease. The techniques used are smear microscopy and culture, but these have the disadvantage of low sensitivity and specificity in skin samples. However, through molecular techniques it is intended to overcome the limitations of conventional methods.

The GenoType ® Mycobacterium Common mycobacteria/additional species (CM/AS) assay (Hain Lifescience; Nehren Germany), is a commercial kit that uses the amplification of the gene RRNA 23S, together with the reverse hybridization with probes of specific oligonucleotides immobilized on membrane strips. Altogether the GenoType ® Mycobacterium CM/AS, identifies 31 species of Mycobacterium, including the complex M. tuberculosis, and a wide spectrum of MNT, being a reliable test for the identification of species of Mycobacteria (Lee AS., et al., 2009).

Material and methods

A descriptive, prospective, open, observational and transversal study was carried out. Samples of patients from the Yucatan Dermatological Center with clinical symptoms suggestive of cutaneous mycobacteriosis were included.

The samples were processed in the microbiology laboratory of the "Dr. Hideyo Noguchi" Regional Research Center, all patients signed informed consent letter to participate in the study. Samples included skin aspirates or biopsies of approximately 10 x 4 mm.

Smear and cultivation

All samples were cultured in Löwenstein-Jensen (L-J), for which the aspirates were sown directly, while the biopsies were macerated in a mortar with 2 ml of saline solution. For the sowing in L-J were taken 100 μ L of the supernatant and incubated at 37 °c for 4 weeks, supervising weekly to corroborate growth. The presence of resistant acid-alcohol bacilli was determined by the staining of Ziehl-Neelsen.

DNA extraction

For DNA extraction, a colony of bacterial culture or 400 M L was taken from the sample maceration. The InstanGeneTM Matrix commercial case was used (Bio-Rad Laboratories; Hercules, CA) following manufacturer's instructions.

Polymerase chain reaction

For each of the amplified genes, the following mixture was performed for the reaction: 12.5 μ L of Go Taq ® Green Master Mix 2x (promega Corporation 2800 Woods Hollow Road), 0.5 μ L of each initiator (1 M m), 10 M L of extracted DNA and 1.5 μ L of water free of nucleases to obtain a final volume of 25 M L. For the determination of gender was conducted a PCR aimed at amplifying a region of the gene that encodes for HSP65, which is retained in all Mycobacterium spp., with an expected enlargement size of 439PB, 50 using the first: HSP65 Fw 5 ´-ACC AAC GAT GGT GTG TCC AT-3 ´ and HSP65 Rv 5 ´-CTT GTC GAA gcc CAT ACC CT-3 ´

The conditions for amplification were as follows: 1 cycle at 94 °c for 5 minutes, then 35 cycles of 94 °c for 1 minute, 60 °c for 1 minute, 72 °c for one minute and a final extension cycle of 72 °c for 7 minutes.

ISSN-On line: 2414-8849
ECORFAN® All rights reserved.

For the determination of the M. Tuberculosis complex is amplified a region of the gene RpoB that encodes the beta subunit of the RNA polymerase, with an expected magnification size of 230 Pb, using the first: MTC Fw 5 '-TAC GGT CGG CGA GCT GAT CCA AA-3 ' and MTC Rv 5 '-ACA GTC G GC GCT TGT GGG TCA AC-3 '. The conditions of cycling for the detection of the species are: 1 cycle at 94 °c for 5 minutes, then 35 continuous cycles will be repeated at 94 °c for 1 minute, 55 °c for 90 seconds and 72 °c for 1 minute, at the end 1 cycle at 72 °c for 5 minutes.

To determine the species M. leprae, sequences directed to amplify a conserved region of the folp1 gene found in the species M. leprae with an expected size of 281 bp were used. using the primers: Fw 5'-GCTTCTCGTGCCGAAGCG-3 'and Rv 5'-CCATCGCGGGATCTGCTCGCCC-3'. Cycling conditions were: 1 cycle at 94 ° C for 5 min, then 35 continuous cycles of 94 ° C for 1 min, 62 ° C for 1 min, 72 ° C for one min, at the end of one cycle at 72 ° C for 7 min and left at 4 ° C.

GenoType® Mycobacterium CM / AS.

For the identification of mycobacterial species, the GenoType® Mycobacterium CM / AS kit was used, following the manufacturer's instructions and using the reagents provided by it. The complete protocol consists in the amplification of a sequence of the 23S rRNA gene (specific for the genus Mycobacterium), for which a BIO-RAD iCyclerTM thermocycler was used, the reaction was carried out as follows: 12.5 µl of Go Taq ® Green Master Mix 2X; 0.5 µl of each primer; 5 µl of extracted DNA and 6.5 µl of nuclease-free water, the amplification conditions were those suggested by the manufacturer. Once the DNA was amplified, the hybridization of the amplification products to specific oligonucleotides immobilized in the membrane strips continued, the development of the strips and the interpretation of the results was carried out according to the manufacturer's instructions.

Results

We included 40 samples from patients with clinical symptoms suggestive of mycobacteriosis, of which only 15 were positive for mycobacteria (37.5%).

Of all the samples, only 8 grew in the L-J culture and had a positive sputum smear (20%) (Figures 1 and 2).

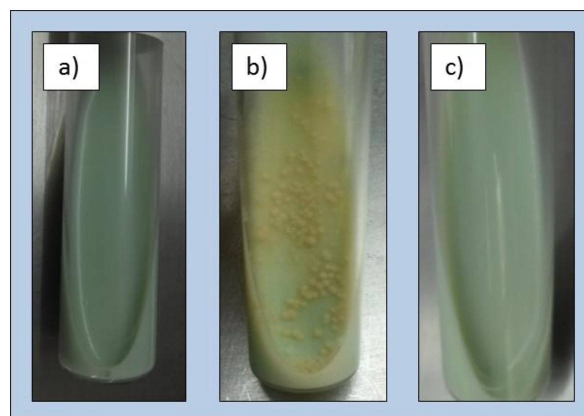

Figure 1 Culture results in Löwenstein-Jensen. a) negative control. b) positive culture. c) negative culture

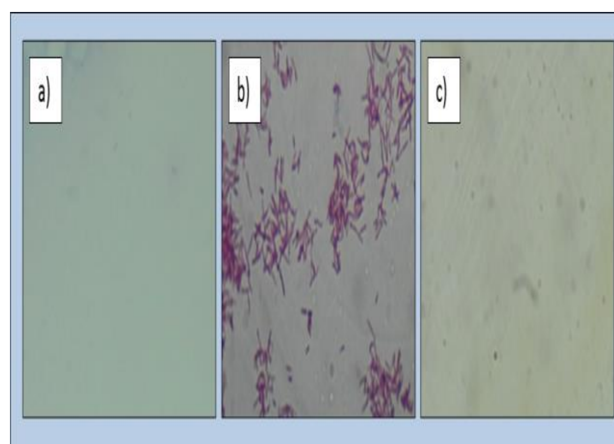

Figure 2 Results of smear microscopy. a) negative control. b) positive Bk c) negative Bk

Of the 40 samples, 15 were positive to the Mycobacterium genus when the hsp65 gene was amplified, however, all were negative to the rpoB gene, which corresponds to the Mycobacterium tuberculosis complex, indicating that they were non-tuberculous mycobacteria.

To the 15 positive samples to Mycobacterium genus, the identification of species was made, using the GenoType® Mycobacterium CM / AS, obtaining as a result that 7 of them only corresponded with the Mycobacterium genus, while 5 of them corresponded with M. fortuitum and 3 with M. abcessus (Figure 3).

ECORFAN® All rights reserved.

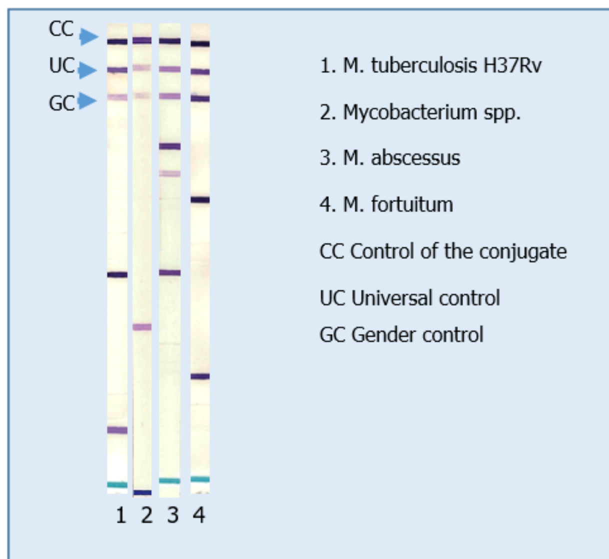

Figure 3 Results of GenoType Mycobacterium CM / AS. 1. In all the strips, the internal controls corresponding to lines 1, 2 and 3 appear; 1. Control of M. tuberculosis strain H37Rv (lines 10 and 16); 2. Mycobacterium spp. (line 12); 3. M. abscessus (lines 5, 6 and 10); M. fortuitum (lines 7 and 14)

The 8 samples that were not identified with the GenoType® Mycobacterium CM / AS, were amplified for the folp1 gene, which corresponds to the species M. leprae, and coincided with the symptoms of the patients.

At the end of the study, of the 40 samples, 15 were positive for mycobacteria, finding the following species: 5 M. fortuitum, 3 M. abcessus and 7 M. leprae.

Discussion and conclusion

PCR resulted in a higher positivity rate for Mycobacterium spp. than culture and smear microscopy. This may be due to the low number of bacteria present at the time of staining; in this regard, other studies indicate that $\geq 10^4$ bacteria per ml are required to obtain a positive result in Ziehl-Neelsen (Almaguer, J., et al 2009). In relation to the higher sensitivity of the PCR, it could be explained by the identification of mycobacterial DNA in samples with a negative result by smear microscopy (Suárez, M. J., et al., 2010). On the other hand, it is known that the culture requires 10 bacilli for isolation, considerably smaller than those suggested for smear microscopy (Parimango, D., et al., 2007). The sensitivity of the PCR in relation to the culture, which is considered the reference method for the diagnosis of tuberculosis, was 87.5%, culture 25% and baciloscopy 12.5%.

The positive results that were obtained by PCR in this study can be due to several factors, including the number of bacilli present in the sample that can give negative results for the culture and smear microscopy. In other cases, patients may have received treatment that would result in the death of the bacilli and would cause the absence of viable bacilli that limit the growth and visualization of colonies in the cultures (Frankel, A., et al., 2009; Tincopa , OW; Sánchez, LS 2003)

The results of this study confirm the importance of molecular techniques in the diagnosis of cutaneous tuberculosis (TB), especially for the correct identification of MNT and M. leprae, since conventional methods have significant disadvantages, which directly affect the treatment inadequate (Frankel, A., et al., 2009; Kandola, P., Meena, L. 2014; Silva, C., et al., 2007).

In this work, it was possible to identify M. fortuitum, M. abcessus with the help of GenoType® Mycobacterium CM / AS, which are opportunistic mycobacteria associated with skin infections, although their pathogenicity is lower than M. tuberculosis, demonstrating that it is a Useful tool for the diagnosis of MNT (Maroñas, L.; Postigo, M. 2013; Yang M., et al., 2016).

M. leprae was also found in patient samples and because this disease is still valid in the state of Yucatan, an accurate and timely diagnosis is important (Cenaprece 2012).

In conclusion, the use of molecular biology techniques for the identification of MNT in patients with TB, is of vital importance, since it guarantees an accurate diagnosis and an adequate treatment.

References

Almaguer, J.; Ocampo, J.; Rendón, A. 2009. Panorama actual en el diagnóstico de la tuberculosis cutánea. Revista española de cardiología; 100, 7, 562-570.

ECORFAN® All rights reserved.

Amresh Kumar Singh, Rungmei S. K. Marak, Anand Kumar Maurya, Manaswini Das, Vijaya Lakshmi Nag, and Tapan N. Dhole, 2015. "Mixed Cutaneous Infection Caused by Mycobacterium szulgai and Mycobacterium intermedium in a Healthy Adult Female: A Rare Case Report," Case Reports in Dermatological Medicine; Article ID 607519, 4 pages,. doi:10.1155/2015/607519

Cassidy PM, Hedberg K, Saulson A, McNelly E, Winthrop, KL. 2009. Nontuberculous mycobacterial disease prevalence and risk factors: a changing epidemiology. Clinical Infectious Diseases; 49(12):124-129.

Frankel, A.; Penrose, C.; Emer, J. 2009. Cutaneous Tuberculosis A Practical Case Report and Review for the Dermatologist. JCAD; 2, 10, 19-27.

Glapinski J; Walkiewicz R.; Safianowska A.; Grubek-Jaworska H. 2004. Computerized Mycobacterium Type Recognition Using Numerical Data Base of Mycolic Acids Elution Profiles. Biocybernetics and Biomedical Engineering;24(1):25-38.

Kandola, P.; Meena, L. 2014. Extrapulmonary tuberculosis: Overview, manifestations, diagnostic and treatment techniques. Adv. Mater. Rev. 1, 1, 13-19.

Lee AS, Jelfs P, Sintchenko V, & Gilbert GL. 2009. Identification of non-tuberculous mycobacteria: utility of the GenoType Mycobacterium CM/AS assay compared with HPLC and 16S rRNA gene sequencing. Journal Of Medical Microbiology;58(7):900-904.

Maroñas, L.; Postigo, M. 2013. Micobacterias cutáneas: un reto diagnóstico. Más Dermatol; 19, 5-13.

Parimango, D.; Chávez, M.; Lujan, M.; Otiniano, M.; Muñoz, E. 2007. Comparación de los métodos Ogawa y Löwenstein-Jensen en el aislamiento de Mycobacterium tuberculosis pulmonar. Hospital Regional Docente de Trujillo, Perú. Rev. Med. Vallejiana; 4, 1. 24-31.

Silva, C.; Bermúdez, V.; Arraiz, N.; Bermúdez, F. 2007. Fármacos de primera línea utilizados en el tratamiento de la tuberculosis. Archivos Venezolanos de Farmacia y Terapéutica; 26,1.

Situación actual y desafíos de la lepra en México. 2012. Disponible en: http://www.cenaprece.salud.gob.mx/programas/interior/micobacteriosis/descargas/pdf/situacionactual_manualprocedimientos.pdf

Suárez, M. J.; Quiroz, J.; Jiménez, E.; Salazar, L. 2010. Diagnóstico molecular de Mycobacterium tuberculosis en cortes histológicos embebidos en parafina: investigación exploratoria. REVISTA MÉDICA DE LA UNIVERSIDAD DE COSTA RICA; 4, 1, 68-78.

Tincopa, O. W.; Sánchez, L. S. 2003. Tuberculosis cutánea. Dermatología Peruana; 13, 3, 195-214.

Yang M, Huh HJ, Kwon HJ, Kim JY, Song DJ, Koh WJ, Ki CS, Lee NY. 2016. Comparative evaluation of the AdvanSure Mycobacteria GenoBlot assay and the GenoType Mycobacterium CM/AS assay for the identification of non-tuberculous mycobacteria. J Med Microbiol. Dec;65(12):1422-1428. doi: 10.1099/jmm.0.000376.

Yew WW, Sotgiu G, Migliori GB. 2010. Update in tuberculosis and nontuberculous mycobacterial disease. American Journal Of Respiratory And Critical Care Medicine;184(2):180-185.

ISSN-On line: 2414-8849
ECORFAN® All rights reserved.