RAPD discloses high molecular diversity of *Mycobacterium tuberculosis* from Michoacán, Mexico

A.L. Guillén-Nepita[1,3], G. Vázquez-Marrufo[1], F.T. Blanco-Guillot[2], G.A. Figueroa-Aguilar[2], M.S. Vázquez-Garcidueñas[3]

[1]Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina;Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo;
[2]Laboratorio Estatal de Salud Pública de Michoacán;
[3]División de Estudios de Posgrado de la Facultad de Ciencias Médicas y Biológicas "Dr. Ignacio Chávez", Universidad Michoacana de San Nicolás de Hidalgo. Morelia, Michoacán, Mexico

SUMMARY

Random amplified polymorphism DNA (RAPD) is an easy, inexpensive technique for the characterization of pathogens in low-income countries. In this study we used RAPD to assess the genetic diversity of a small collection of isolates of mycobacteria from the Mexican state of Michoacán. In contrast with the low annual tuberculosis incidence in Michoacán relative to the national average, we found a high molecular diversity value suggesting high population diversity of *M. tuberculosis* in the studied region. Our findings justify further typing efforts with other molecular tools such as MIRU-VNTR and spoligotyping.

KEY WORDS: Mycobacterium tuberculosis, RAPDs, Mexico, Genetic diversity.

Received March 04, 2013 *Accepted* June 14, 2013

In 2010, the tuberculosis (TB) incidence in Mexico averaged 16.8 cases per 100,000 inhabitants; Michoacán and Guanajuato reported 7.7 and 7.4 cases per 100,000 inhabitants, respectively (Secretaría de Salud, 2011). Typing of *M. tuberculosis* and molecular epidemiological studies of TB currently rely on IS*6110*-RFLP (van Embden *et al.,* 1993), spoligotyping (Kamerbeek *et al.,* 1997) and MIRU-VNTR (Supply *et al.,* 2006).

These molecular techniques are complex, lengthy and expensive, so the variation of the bacterial genome in low-income settings with a high prevalence of TB can be assessed more simply, quickly and inexpensively by random amplification of polymorphic DNA (RAPD) (Williams *et al.,* 1990).

Corresponding author
Dr. Ma. Soledad Vázquez Garcidueñas
Ave. Rafael Carrillo
esq. Dr. Salvador González Herrejón s/n
Col. Cuauhtémoc, Morelia, Michoacán Mexico - C.P. 58020
E-mail: marisolvaz@yahoo.com

The RAPD technique is one of the molecular tools available for tracking pathogenic bacteria outbreaks (Li *et al.,* 2009). Local studies made in clinic and research laboratories at can rely on RAPD for the characterization of a wide range of bacteria including *Escherichia coli, Salmonella enterica* (Holley *et al.,* 2008; Dione *et al.,* 2012), *Campilobacter ssp* (Adzitey *et al.* 2012), and *M. tuberculosis* (Richner *et al.,* 1997; Tazi *et al.,* 2004; Vázquez-Marrufo *et al.*, 2008; Hashemi *et al.,* 2012). In this work we assessed by RAPD the molecular diversity of a small sample of *Mycobacterium tuberculosis* isolates from prevalent TB cases reported in the states of Michoacán and Guanajuato, Mexico, of which available epidemiological data were examined. The results of this preliminary study justify extending joint efforts with the regional public health authorities for characterization by MIRUS and spoligotyping of a larger number of local strains of *Mycobacterium tuberculosis*.

The studied strains derived from 33 human samples of sputum and extrapulmonary fluids (cere-

brospinal fluid and urine) from the state of Michoacán provided by the State Public Health Laboratory and the General Hospital "Dr. Miguel Silva" between 2009 and 2010, and two sputum samples were obtained from the neighboring state of Guanajuato through the State General Hospital. Epidemiological data were collected anonymously (Table 1). *M. tuberculosis* H37Rv was used as a reference strain. The samples were processed as indicated by the Institute of Epidemiological Diagnosis and Reference (InDRE, http://www.cenavece.salud.gob.mx/indre/interior/lab_micobacterias.html). Isolate species were identified from IS*6110* amplification reports by InDRE. Isolates failing to produce an amplicon were considered mycobacteria other than tuberculosis (MOTT).

The mycobacteria colonies from axenic cultures were inactivated by incubation in 400 µL of lysis buffer (100 mM Tris-HCl pH8, 0.2% SDS, 100 mM NaCl, 50 mM EDTA) at 80°C for 1h, after which they were ready for genomic DNA isolation by the phenol-chloroform method (Vázquez-Marrufo *et al.*, 2008). RAPD reactions were performed in separate PCR assays using 6 different primers: N9, R8, U10, U13 (Tazi *et al.*, 2004), DG93 (Miyata *et al.,* 1995) and DG102 (Chansiripornchai *et al.,* 2000). The reaction mix and the amplification program followed Williams *et al.* (1990).

Amplification products were separated by electrophoresis in 2% agarose gels. Assays were carried out in triplicate and only reproducible bands were taken into account for analysis. The size of the obtained bands was calculated using Quantity One 4.4.1 Software (BIORAD, USA). All bands in the gel below 4K were analyzed by the MATCH function, and a presence/absence binary matrix was built. For comparison purposes, Shannon's index (*H'*) was calculated using $\log_2$, $\log_{10}$ and ln (Shannon, 1948). Simpson's index (D) and dendrograms were obtained from the matrix using PAST software and Ward's method (Hammer *et al.,* 2001).

The epidemiological traits of patients from whom isolates were obtained are listed in Table 1. No epidemiological links were reported between these patients. Diabetes mellitus type II (DM-II) was the most frequent co-morbidity factor, which is consistent with national statistics (Secretaría de Salud, 2009). Only three isolates (8.5%) were derived from extrapulmonary TB patients diagnosed with tuberculous meningitis (TBM).

Through RAPD we identified 84 bands suitable for analysis ranging in size from 370 to 3500 bp, from which 43 were present in less than 10% of the isolates. The dendrogram (Figure 1a, A-C) shows that all 35 isolates and the H37Rv strain can be grouped in 3 main clusters, from which 13 groups of closely related isolates derive (Figure 1a, G1-G13). The D value for the RAPD technique was 0.925, showing the usefulness of RAPD typing, even in cases of clonal organisms such as *M. tuberculosis*.

Shannon's Index is not commonly applied for assessing the diversity of *M. tuberculosis*, except for a single report of Vázquez-Marrufo *et al.* (2008). The Shannon's Index values we calculated were: H'_2=3.4749, H'_n=2.4086 and H'_{10}=1.46 - a value similar to H'_{10}=1.54 reported for an independent set of strains from Mexican patients (Vázquez-Marrufo *et al.,* 2008). These values exceed those reported for human associated *Streptococcus aureus* (H'_2=0.56) (Reinoso *et al.,* 2004), and for phylogenetic subgroups of human *Escherichia coli* (H'_n =0.6598) (Carlos *et al.,* 2010), but resemble those of invasive populations of *Streptococcus pneumoniae* (H'_2 from 1.92 to 3.75) (de la Pedralosa *et al.,* 2009), and environmental *E. coli* populations (H'_n from 2.6191 to 2.9264) (Nelson *et al.,* 2008).

These values also reflect the lack of clustering observed in the dendrogram, and suggest high diversity between isolates. Other researchers in different settings (Richner *et al.,* 1997; Tazi *et al.,* 2004) have also observed high molecular diversity of *M. tuberculosis* isolates. In contrast to the report of Vázquez-Marrufo *et al.* (2008), we were able to examine possible links between epidemiological traits and RAPD patterns. Diverse patterns were found between isolates obtained from patients with common co-morbidity, age or geographical region.

TBM strains, for example, are dispersed throughout the dendrogram (Figure 1a, G4, G5, and G8) as anticipated by the absence of a common index case. Thus, in our region TBM may be caused by more than one strain, as also suggested by regional epidemiological information. Each year since 2008, 1 to 2% (3 to 7 cases) of the reported total annual incidences of TB have been of central nervous system tuberculosis (CNST) includ-

TABLE 1 - *Epidemiological traits of patients included in this study.*

Patient	*Disease location*	*Age*	*Gender*[a]			
1	Pulmonary	57	M	3	None	001, 011
2	Renal/No TB	24	F	1	None	002
3	Meningeal	60	M	7	Cysticercosis	003
4	Meningeal	nd	M	1	None	004
6	Pulmonary	67	F	5	Diabetes	006
7	Pulmonary	37	M	7	Diabetes	007
8	Pulmonary	66	M	5	Alcoholism	008
9	Pulmonary	24	F	5	None	009
10	Pulmonary	88	M	1	None	010
11	Pulmonary	34	M	3	None	012
12	Pulmonary	59	M	3	Alcoholism	013, 014
13	Pulmonary	53	F	1	Diabetes	015
15	Pulmonary	67	M	6	Diabetes	017
16	Pulmonary	29	M	7	None	018
17	Pulmonary	53	F	1	None	019
18	Pulmonary	55	F	7	Diabetes	020
19	Pulmonary	89	M	3	None	021
20	Pulmonary	77	M	1	None	022
21	Pulmonary	Nd	F	Nd	None	023
22	Pulmonary	48	M	2	Diabetes	024
23	Pulmonary	55	F	1	Diabetes	025
24	Meningeal	31	M	1	None	026
25	Pulmonary	19	M	Nd	None	027
26	Pulmonary	87	F	Nd	Diabetes	028
27	Pulmonary	39	M	7	I.V. Drug User	029
28	Pulmonary	29	F	7	None	030
29	Pulmonary	Nd	F	1	None	031
30	Pulmonary	30	F	8	None	032
31	Pulmonary	20	M	7	Malnutrition	033
32	Pulmonary	55	F	Gto	Diabetes	034
33	Pulmonary	Nd	M	Gto	None	035

[a]M, Male; F, Female. [b]1, Morelia; 2, Zamora; 3, Zitácuaro; 4, Pátzcuaro; 5, Uruapan; 6, La Piedad; 7, Apatzingán; 8, Lázaro Cárdenas. GTO, Samples from state of Guanajuato. Nd, no data available.

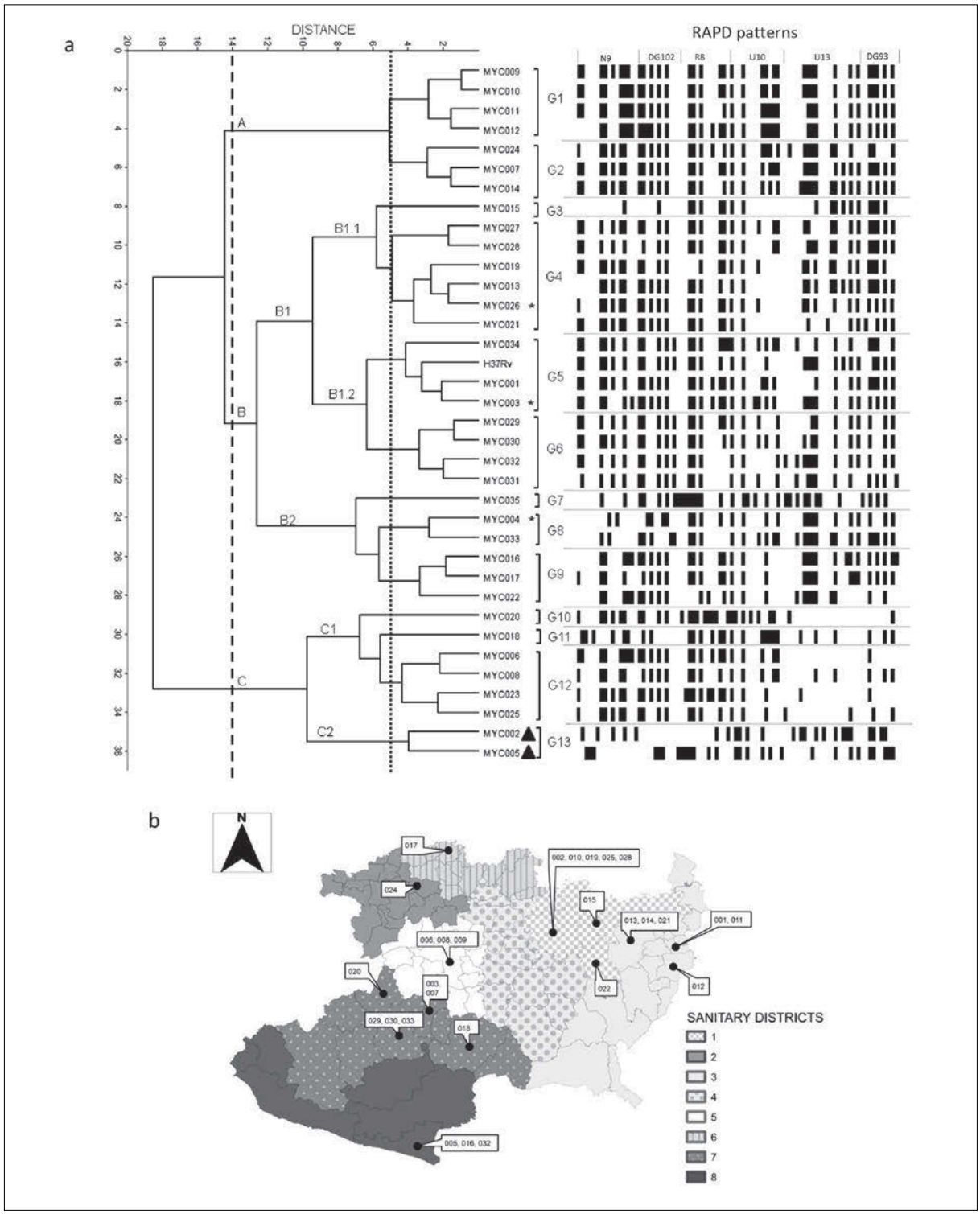

FIGURE 1 - *RAPD - based dendrogram and geographic location of the MYC isolates. a) Dendrogram obtained by Ward's method. Dashed line: distance value of 14. Dotted line: distance value of 5. RAPD patterns are a graphic representation of presence (black area) or absence (white area) of each of the 84 bands obtained across the 6 primers used. (*) TBM isolates. (▲) MOTT isolates. b) Geographic location of MYC strains from Michoacán.*

ing TBM (SINAVE, 2012), all of which have occurred dispersed throughout the state of Michoacán. Such incidence rates and the dispersed geographical distribution of TBM cases agree with our RAPD results.

Therefore, we are interested in this seemingly diverse mycobacterial population, particularly within the TBM isolates. Further typing of a larger strain sample including those from the present study is currently being made. We emphasize that the use of a fast, inexpensive technique such as RAPD may reveal interesting data from isolate collections and is useful as a preliminary tool to direct research to fruitful directions.

ACKNOWLEDGEMENT

This study was supported by Grant 16.4 from Coordinación de la Investigación Científica-U.M.S.N.H. ALGN is indebted to Doctoral fellowship from CONACYT.

REFERENCES

Adzitey F., Rusul G., Huda N., Cogan T., Corry J. (2012). Prevalence, antibiotic resistance and RAPD typing of Campylobacter species isolated from ducks, their rearing and processing environments in Penang, Malaysia Int. *J. Food Microbiol.* **154**, 197-205.

Dione M.M., Geerts S., Antonio M. (2012). Characterisation of novel strains of multiply antibiotic-resistant Salmonella recovered from poultry in Southern Senegal *J. Infect. Dev. Ctries.* **6**, 436-442.

Carlos C., Pires M.M., Stoppe N.C., Hachich E.M., Sato M.I., Gomes T.A., Amaral L.A., Ottoboni L.M. (2010). *Escherichia coli* phylogenetic group determination and its application in the identification of the major animal source of fecal contamination. *BMC Microbiol.* **10**, 161.

Chansiripornchai N., Ramasoota P., Bangtrakulnonth A., Sasipreeyajan J., Svenson, S.B. (2000). Application of randomly amplified polymorphic DNA (RAPD) analysis for typing Avian *Salmonella enterica* subsp. enterica. FEMS Immunol. *Med. Microbiol.* **29**, 221-225.

de la Pedrosa E.G., Baquero F., Loza E., Nadal-Serrano J.M., Fenoll A., Del Campo R., Cantón R. (2009). High clonal diversity in erythromycin-resistant *Streptococcus pneumoniae* invasive isolates in Madrid, Spain (2000-07). *J. Antimicrob. Chemother.* **64**, 1165-1169.

Hammer O., Harper D.A.T., Ryan P.D. (2001). PAST: Paleontological Statistics software package for education and data analysis. Paleontol. *Electron.* **4**, 9.

Hashemi A., Shojaei H., Heidarieh P., Aslani M. M., Naser A.D. (2012). Genetic diversity of Iranian clinical isolates of *Mycobacterium tuberculosis*. *New Microbiol.* **35**, 61-65.

Holley R., Walkty J., Blank G., Tenuta M., Ominski K., Krause D., Ng L.K. (2008). Examination of *Salmonella* and *Escherichia coli* translocation from hog manure to forage, soil, and cattle grazed on the hog manure-treated pasture. *J. Environ. Qual.* **37**, 2083-2092.

Kamerbeek J., Schouls L., Kolk A., van Agterveld M., van Soolingen D., Kuijper S., et al. (1997). Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J. Clin. Microbiol.* **35**, 907-914.

Li W., Raoult D., Fournier P.E. (2009). Bacterial strain typing in the genomic era. *FEMS Microbiol.* ***Rev.*** **33**, 892-916.

Miyata M., Aoki T., Inglis V., Yoshida T., Endo M. (1995). RAPD analysis of *Aeromonas salmonicida* and *Aeromonas hydrophila*. *J. App. Bacteriol.* **79**, 181-185.

Nelson M., Jones S.H., Edwards C., Ellis J.C. (2008). Characterization of *Escherichia coli* populations from gulls, landfill trash, and wastewater using ribotyping. *Dis. Aquat. Organ.* **81**, 53-63.

Reinoso E., Bettera S., Frigerio C., DiRenzo M., Calzolari A., Bogni C. (2004). RAPD-PCR analysis of *Staphylococcus aureus* strains isolated from bovine and human hosts. *Microbiol. Res.* **159**, 245-255.

Richner S., Meiring J., Kirby R. (1997). A study of the genetic diversity of *Mycobacterium tuberculosis* isolated from patients in the Eastern Province of South Africa using random amplified polymorphic DNA profiling. *Electrophoresis.* **18**, 1570-1576.

Secretaría de Salud. (2009). Estándares para la Atención de la Tuberculosis en México. Mexico City.

Secretaría de Salud. (2011). Situación Actual de la Tuberculosis en México. Avances y Desafíos. Mexico City.

Shannon C.E. (1948). A Mathematical Theory of Communication.The Bell System *Technical Journal.* **27**, 379-423.

SINAVE (2012). Sistema Nacional de Vigilancia Epidemiológica. http://www.sinave.gob.mx Accesed May 15th 2013.

Supply P., Allix C., Lesjean S., Cardoso-Oelemann M., Rüsch-Gerdes S., Willery E., et al. (2006). Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **44**, 4498-4510.

Tazi L., El Baghdadi J., Lesjean S., Locht C., Supply P., Tibayrenc M., Bañuls A.L. (2004). Genetic diversity and population structure of *Mycobacterium tu-*

berculosis in Casablanca, a Moroccan city with high incidence of tuberculosis. *J. Clin. Microbiol.* **42**, 461-466.

VAN EMBDEN J.D., CAVE M.D., CRAWFORD J.T., DALE J.W., EISENACH K.D., GICQUEL B., ET AL. (1993). Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* **31**, 406-409.

VAN BELKUM, VAN PELT-VERKUIL E., HAYS J.P. (2009). The application of randomly amplified DNA analysis in the molecular epidemiology of microorganisms. In D.A. Caugant (ed.) Molecular Epidemiology of Microorganisms, Methods in Molecular Biology. Vol. 55. Humana Press, New York USA. 37-46.

VÁZQUEZ-MARRUFO G., MARÍN-HERNÁNDEZ D., ZAVALA-PÁRAMO M.G., VÁZQUEZ-NARVÁEZ G., ÁLVAREZ-AGUILAR C., VÁZQUEZ-GARCIDUEÑAS M.S. (2008). Genetic diversity among *Mycobacterium tuberculosis* isolates from Mexican patients. *Can. J. Microbiol.* **54**, 610-618.

WILLIAMS J.G., KUBELIK A.R., LIVAK K.J., RAFALSKI J.A., TINGEY S.V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18**, 6531-6535.