SHORT COMMUNICATION

IS6110 Fingerprinting of Sensitive and Resistant Strains (1991-1992) of *Mycobacterium tuberculosis* in Colombia

Jorge Enrique Gómez-Marin/+, Clara Inés León Franco*, Martha Inirida Guerrero*, Leen Rigouts, Françoise Portaels****

Centro de Investigaciones Biomedicas, Facultad de Medicina, Universidad del Quindio, Armenia (Q), Colombia *Laboratorio de Micobacterias, Subdirección de Investigación y Desarrollo, Instituto Nacional de Salud, Bogotá, Colombia **Laboratory of Mycobacteriology, Institute of Tropical Medicine, Antwerpen, Belgium

The standardized method to study the polymorphism of IS 6110 was used to characterize 53 isolates of Mycobacterium tuberculosis *obtained during 1991-1992 from 14 regions in Colombia. In Valle region cluster rate was 25% (4/16). The mean number of IS6110 band was 10 ± 3. Similarity between strains was of 60% in 81% of strains and this tended to be correlated with geographic origin. For the first time* M. tuberculosis *without IS6110 bands in restriction fragment length polymorphism analysis was found in Colombia. Additional studies are necessaries in order to best characterize the situation in relation to human immunodeficiency virus epidemic and recent changes in tuberculosis control program.*

Key words: IS6110 - *Mycobacterium tuberculosis* - Colombia

In Colombia are reported near of 11,000 new cases of tuberculosis each year (Victoria 1999). The incidence of new cases has been shown to be in decrease, however this can be explained by a reduction in the search of new case (Victoria 1999). There are an urgent need to best define effective measures in order to stop transmission in particular conditions where restriction fragment length polymorphism (RFLP) analysis can plays a helpful role. RFLP is based on the detection of the species specific insertion sequence IS 6110 that allows differentiation of strains belonging to the *Mycobacterium tuberculosis* complex (van Soolingen et al. 1991). IS6110 is stable enough to provide identical fingerprints for mycobacterial strains isolated from one patient and from patients belonging to small clusters of infection (van Soolingen et al. 1991). Most studies with IS 6110 fingerprinting have focused on tracing routes of transmission of *M. tuberculosis* in outbreaks. The expanded use of fingerprinting have identified sources of infection and routes of transmission that were previously not known or suspected (Crawford et al. 1993, Geneiwen et al. 1993). Findings of this sort could help in developing certain types of policies or actions to more effective control measures. In addition, some data have been published and indicate that there are an association between fingerprinting types and their geographic origin (Hermans et al. 1995). These studies have shown that it is possible to distinguish between isolates originating from different locations.

+Corresponding author. Fax: +57-67-460168. E-mail : jegomezmarin@hotmail.com
Received 19 December 2001
Accepted 7 August 2002

Screening of all isolates from a particular geographical area would identify related isolates and provide the starting point for contact investigations. In 1993, international consensus was reached on a standardized method of *M. tuberculosis* RFLP analysis opening the way to interlaboratory comparison (van Embden et al. 1993). However, in a Medline search using MESH term IS6110, appeared 77 papers dealing with use of RFLP for IS6110 in Europe, 103 from United States, 35 from Africa and only 17 articles from Latin America. Some papers from Latin America characterized the polymorphims in general and human immunodeficiency virus (HIV) population (Escalante et al. 1998, Ferrazoli et al. 2000, Suffys et al. 2000, Diaz et al. 2001, Yang et al. 2001). In Colombia, two previous published works (Gomez-Marin et al. 1995, Henao et al. 1999) reported RFLP patterns of IS6110 in *M. tuberculosis* strains in two particular regions: Quindio (center west region department) and Guaviare (eastern region department). The objective of the present work was to enlarge and complete these previous studies and to characterize IS6110 polymorphism of *M. tuberculosis* strains obtained in different geographical areas in Colombia. The group was a sample of 53 from a total of 121 strains collected during a survey on initial drug resistance in different regions of Colombia performed at the National Institute of Health of the Republic of Colombia during 1991-1992. All the patients were smear positive. The methodology used to select this group of patients has been described in detail previously (Laszlo & Kantor 1995).

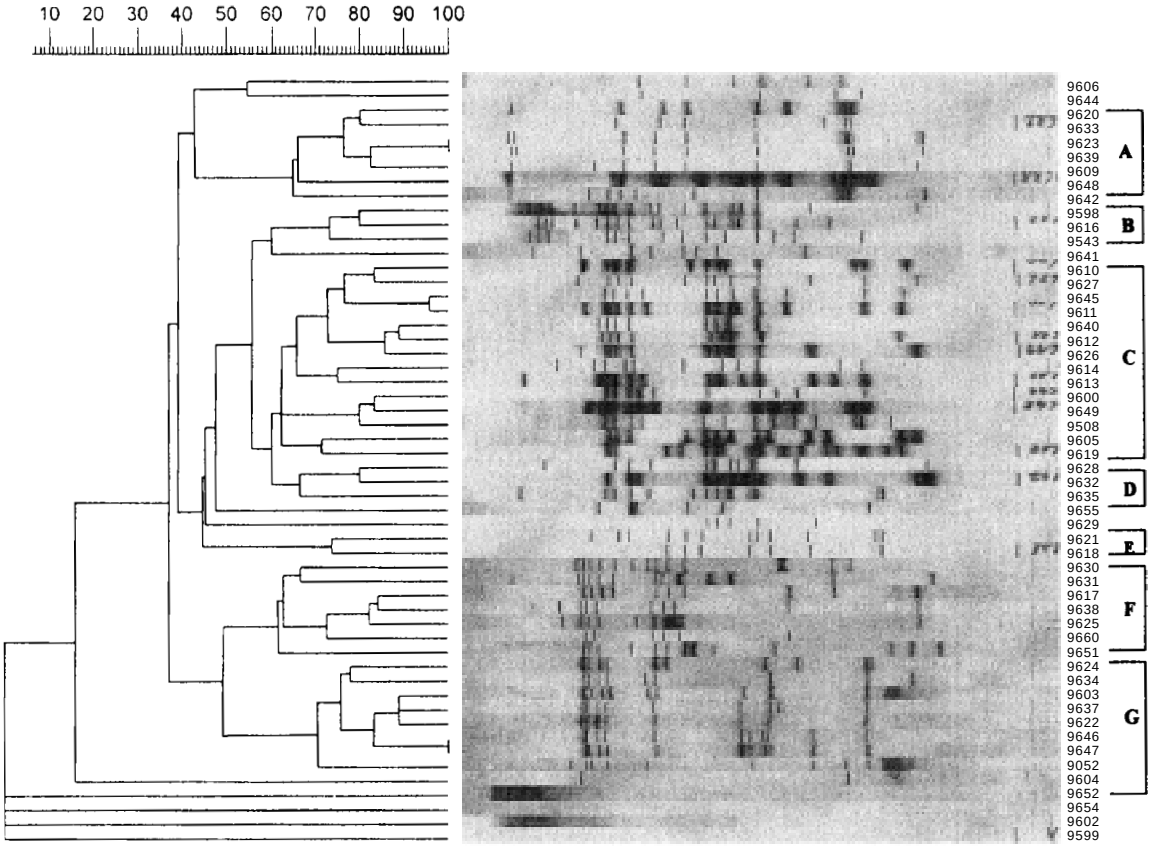
Samples were decontaminated using the Kudoh's method and cultured on Kudoh's modified Ogawa medium and Giraldo's modified Stonebrink medium at 37°C (Orozco et al. 1985). Culture identification was made by the CDC's criteria (Kent & Kubric 1985) and drug susceptibility testing by the proportion method of Cannetti et al. (1969) for isoniazide (INH, 0.2 µg/ml), rifampicine (RPM,

40 µl/ml), ethambutol (EMB, 2 µg/ml) and streptomicine (SM, 4 µg/ml). Resistance was defined according to the past history of treatment referred by the patient. Some of the results of the drug sensitivity testing were confirmed in the WHO-Colaborator Center in Ottawa (Canada) or in the Institute of Tropical Medicine in Antwerpen. Strains were sent to the Institute in Antwerpen on L-J medium for fingerprinting.

The standardized method (van Embden et al. 1993) was applied to all isolates. Briefly about 10 mg of bacteries were suspended in 500 µl TE buffer (10 mM, Tris HCl pH 8.0, 1 mM EDTA). After lysis with lysozyme, proteinase K and SDS (Sodium Dodecyl Sulfate), the DNA was extracted with CTAB (cetyltrimethylammoniumbromide). The DNA was digested with *Pvu*II, and the fragments were separated by electrophoresis on a 0.8% agarose gel followed by a DNA transfer to a charged nylon membrane by vacuum-blotting. The membrane was hybridized with a 245 bases pair long probe which is directed against the segment at right of cut site of *Pvu*II in the IS6110. The detection of the peroxidase labeled probe was realized by the Enhanced Chemoluminiscence System (ECL, Amersham, UK) and registered on hypersensitive films. To obtain accurate standardization of RFLP results two internal molecular weight markers (supercoiled DNA ladder/*Pvu*II and *PhiX174Hae*III) were included in each sample and two external markers on each membrane. The last consisted of lambda*Hind*III to check the migration during electrophoresis and DNA of Mt14323 *M. tuberculosis* reference strain with a known fingerprint. These internal markers were used during the computer assisted analyses.

The DNA fingerprint patterns of the mycobacterial analysis were analyzed and compared by a computer assisted system (Gelcompar 4.2 software, Kortrijk, Belgium) in order to construct dendrograms based on similarity coefficients. IS6110 groups were formed based on a similarity coefficient greater than 60%.

In total, 53 isolates of *M. tuberculosis* derived from 53 unrelated patients from 14 regions in Colombia were studied. The results of IS6110 characterization are shown in the Figure. We found that two couples of isolates from Valle region had identical IS6110 pattern. Thus, 25% (4/16) of the strains in Valle department originated two clusters that probably were originated from two index cases of recent transmission. However, we were unable to obtain the epidemiological data concerning these cases. By using the criteria of similarity coefficient greater than 60% it were formed seven groups of IS6110 related strains (Figure). The distribution of groups by department and type of resistance is shown in the Table. Group A have a large number of strains located in western departments (Valle, Risaralda and Antioquia), only one isolate from this group was obtained in Caribbean region. Also, a large group of strains from Antioquia were in group F. In total, 43 of 53 isolates (81%) were grouped based on this similarity coefficient. No correlation was found between RFLP types and the phenotypes of resistance to various antitu-



Computer generated lane map and dendrogram based on computer assisted comparison of DNA fingerprints obtained by IS6110 probes from 53 isolates of *Mycobacterium tuberculos*is of 14 regions in Colombia.

berculous drugs. Each group included resistant and sensitive isolates.

The mean number in all strains that showed some IS6110 band was 10 ± 3. The number of copies ranged of 0 to 18. Four isolates were found without IS6110 copies and were originating from four different regions in Colombia (Antioquia, Valle, Choco and Tolima). The number of isolates with low number of copies (less than 4) was 6 of 53 isolates studied (11%).

The two clusters of strains with identical fingerprinting in present study occurred in Valle, the region with the greater number of isolates included. The percent of isolates that were related with probably recent transmission (25%) in Valle strains is near to the 14% found in Quindio (Gómez-Marin et al. 1995). However, the percentage is in contrast with the rate found in Guaviare where were found 31 isolates that formed cluster between 55 isolates (56%) that were studied (Henao et al. 1999). The situation in Gauviare is particular because most of cases were originated from indigenous people that have a rate of incidence of 558 cases/100.000 habitants, a frequency 100 times greater than in non-indigenous population.

Dendrograms are used to establish IS6110 degrees of similarity between isolates. Thus, in a study in Tanzania 45% of non-identical isolates were included in three big groups with similarity coefficients that ranged between 70 to 96 % (Yang et al. 1995) and in Tegucigalpa (Honduras) where 30% of non identical isolates have similarity coefficients greater than 90% with an incidence of tuberculosis (TB) of 84/100,000 habitants (Pineda-Garcia et al. 1997). We selected a coefficient of similarity greater than 60% to establish groups of IS6110 patterns. Although the stability of IS6110 element is high, it is by nature transposable and the highly related but non identical patterns reflect the result of evolution of IS6110 through generations of tuberculosis infections in the country. When groups of isolates were located geographically, it was observed that in some groups DNA fingerprint patterns tended to be in accordance with geographical origins of the isolates. This was, obviously, more evident in groups formed by a number of isolates greater than two or three. Thus, in Antioquia region 5 of 11 isolates (45%) were classed in Group F. This is evidence that coefficient similarity can be used to reflect the common past of IS6110 exchange between diverse migratory and selective forces. These factors can acts as positive selection factors. Evolutionary forces in Colombia can include, among others, people of diverse ethnic origin, migratory movements and a long history of vaccination with BCG vaccines that is compulsory in our country in all newborns. In this context, it will be important to compare results of present study performed during 1992 and isolates of recent years where national BCG production and rates of vaccination have been reduced.

The number of IS6110 copies was evenly distributed within a range of 0 to 18. The most frequent number of bands was 9. Notoriously, for the first time in Colombia we found some isolates without IS6110 bands. The total of isolates with low number of copies (less than 4) was 11%, that is a percent intermediate between regions of the world with an important proportion of isolates with absence or low number of copies as 21% in Vietnam (Yuen et al. 1993) or 24% in Guadeloupe, French West Indies (Sola et al. 1997) or 26% in Tanzania (Yang et al. 1995) and regions with only 8% in Denmark (Yang et al 1994) or 5% in Tunisia (Hermans et al. 1995) or 4% in Cuba (Diaz et al 2001). It have been showed for others authors that isolates with IS6110 lower than four require the use of additional genetic markers, such as PGRS and DR, to establish the genetic relatedness (van Soolingen et al. 1993). It

TABLE

Geographic origin of 53 Colombian strains *Mycobacterium tuberculosis* isolated at the "Instituto Nacional Salud" (Bogotá, 1991)

Department	TB incidence X 100.000 [a]	Number of strains	Code (resistance) [b]	Cluster group of IS6110 [c]
Choco	38	1	9602	-
Tolima	25	2	9598 (PZ), 9599	B (1)
Guajira	59	2	9605 (PZ), 9613	C (1)
Cesar	30	2	9603 (SM-PZ), 9606	G (1)
Quindio	36	1	9608 (PZ)	C (1)
Valle	19	16	9635 (PZ), 9620, 9621, 9623, 9639, 9640, 9643, 9644, 9642, 9641, 9655 (EMB), 9634, 9622, 9646, 9647, 9654	A (4), C (1), D (1), E (1), G (4)
Risaralda	22	4	9645, (PZ) 9629, 9606, 9628	A (1), D (1)
Cáqueta	38	1	9614	-
Santander	19	1	9600	C (1), G (1)
Bolívar	10	2	9610, 9618	C (1), E (1)
N. Santander	36	5	9611, 9612, 9624, 9625, 9626	C (3), F (1), G (1)
Atlántico	35	3	9616, 9619 (SM-INH), 9630	B (1), C (1), F (1)
Huila	32	2	9604 (SM-INH-EMB-RM), 9627	C (1)
Antioquia	15	11	9632, 9633, 9648 (SM), 9643, 9631, 9617, 9638 (SM-INH), 9650, 9651, 9637, 9652	A (2), D (1), F (5), D (1)

a: obtained from Victoria et al . (1999); *b*: resistance patterns in parenthesis; SM: streptomycin, PZ: pirazinamide, EMB: ethambutol, RM: rifampin, INH: isoniazid; *c*: determined by the clustering of IS6110 polymorphisms as showed in the Figure. The number of strains is indicated in parenthesis; TB: tuberculosis

seems that *Pvu*II site of restriction in IS6110 can be methyled in some strains and this can explains that can be missed during Southern blotting (van Soolingen et al. 1996).

In conclusion, this study showed that similarity between strains was of 60% in 81% of strains and this tended to be correlated with geographic origin. However, due to the small number of strains studied a new work is needed to include a representative number of strains from all isolates that are found each year in Colombia. Also it would be necessary to complement epidemiological investigation of cases with identical fingerprinting that we were unable to perform due to the retrospective character of present work. For the first time *M. tuberculosis* without IS6110 bands in RFLP was found in Colombia and 11% of isolates had less than four bands. Additional studies are necessaries in order to characterize the situation in relation with HIV epidemic and recent changes in tuberculosis control program as the reduction in BCG vaccination.

REFERENCES

Cannetti G, Wallace Fox A, Khomenko A, Mahler HT, Menon MK, Mitchison DA, Rist N, Ämelov NA 1969. Advances in techniques of testing mycobacterial drug sensitivity and the use of sensitivity test in tuberculosis control programs. *Bull WHO 41*: 21-43.

Crawford JT 1993. Applications of molecular methods to epidemiology of tuberculosis. 9th Forum in Microbiology. *Res Microbiol 144*: 111-115.

Diaz R, Gomez R, Restrepo E, Rumbault R, Sevy Court J, Valdivia JA, van Soolingen D 2001. Transmission of tuberculosis in Havana, Cuba. A molecular epidemiological study by IS6110 restriction polymorphism typing. *Mem Inst Oswaldo Cruz 96*: 437-443.

Escalante P, Ramaswanny S, Sanabria H, Soini H, Pan X, Valiente O, Musser JM 1998. Genotypic characterization of drug resistant *Mycobacterium tuberculosis* isolates from Peru. *Tuber Lung Dis 79*: 111-118.

Ferrazoli L, Palaci M, Marques LR, Jamal LF, Afiume SB, Chimara E, Martins MC, Silva MA, Oliveira CA, Pelhares MC, Spada DT, Riley LW 2000. Transmission of tuberculosis in an endemic urban setting in Brazil. *Int J Tuber Lung Dis 4*: 18-25.

Genewein A, Telenti A, Bernasconi C, Mordasini C, Weiss S, Maurer AM, Rieder HL, Schopfer K, Bodmer T 1993. Molecular approach to identifying route of transmission of tuberculosis in the community. *Lancet 342*: 841-844.

Gómez-Marin JE, Rigouts L, Villegas LE, Portaels F 1995. Restriction fragment length polymorphism (RFLP) analysis and tuberculosis epidemiology. *Bull PAHO 29*: 226-236.

Henao G, de la Hoz F, León CI, Ribón W, Guerrero MI 1999. Epidemiología clásica y molecular de la tuberculosis en Guaviare 1997-1998. *Informe Quincenal Epidemiológico Nacional 4*: 85-91.

Hermans PWM, Messadi F, Guebrexabher H, van Soolingen D, de Haas PEW, Heersma H, de Neeling H, Ayoub A, Portaels F, Frommel D, Zribi M, van Embden JDA 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia and the Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. *J Infect Dis 171*: 1504-1513.

Kent P, Kubica G 1985. *Public Health Mycobacteriology: A Guide for The Level III Laboratory*, Center for Diseases Control, Atlanta.

Laslo A, Kantor IN 1995. Encuesta por muestreo aleatorio de farmacorresistencia inicial en casos de tuberculosis en America Latina. *Bol Oficina Sanit Panam 119*: 226-235.

Orozco LC, Quintero de R O, Ulloa de M AI, Giraldo de BE, León CI, Naranjo N, Bernal M, Guerrero MI 1985. *Tuberculosis: Manual de procedimientos*, Red Nacional de Laboratorios, Instituto Nacional de Salud, Serie de Publicaciones Científicas No. 1, Bogotá.

Pineda-Garcia L, Ferrera A, Hoffner SE 1997. DNA fingerprinting of *Mycobacterium tuberculosis* strains from patients with pulmonary tuberculosis in Honduras. *J Clin Microbiol 35*: 2393-2397.

Sola C, Horgen L, Seng- Goh K, Rastogi N 1997. Molecular fingerprinting *of Mycobacterium tuberculosis* on a Caribbean island with IS6110 and DR probes. *J Clin Microbiol 5*: 843-846.

Suffys PN, Ivens ME, Rossetti ML, Zahab A, Barroso EW, Barreto AM, Campos E, van Soolingen D, Kremer K, Heerma H, Degrave WM 2000. Uselfulness of IS6110 restriction fragment polymorphism typing of Brazilian strains of *Mycobacterium tuberculosis* and comparison with an international fingerprint database. *Res Microbiol 151*: 343-351.

van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM, Small PM 1993. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol 31*: 406-409.

van Soolingen D, de Haas EW, Hermans PW, Groenen PMA, van Embden JD 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentitation and epidemiology of *Mycobacterium tuberculosis*. *J Clin Microbiol 31*: 1987-1995.

van Soolingen D, de Haas P, Blumenthal R Blumenthal RM, Kremer K, Sluijter M, Pijnenburg JE, Schouls LM, Thole JE, Dessens-Kroon MW, van Embden JD, Hermans PW 1996. Host modification of *Pvu*II restriction site in *Mycobacterium tuberculosis*. *J Bacteriol 178*: 78-84

van Soolingen D, Hermans PWM, de Haas PEW, Sol DR, van Embden A 1991. The ocurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of IS dependent DNA polymorphisms as a tool in the epidemiology of tuberculosis. *J Clin Microbiol 29*: 2578-2586.

Victoria JE 1999. Epidemiologia de la tuberculosis en Colombia. *Informe Quincenal Epidemiológico Nacional 4*: 82-85.

Yang ZH, de Haas PEW, van Soolingen D, van Embden JDA, Andersen AB 1994. Restriction fragment length polymorphism of *Mycobacterium tuberculosis* strains isolated from Greenland during 1992: Evidence of tuberculosis transmission between Greenland and Denmark. *J Clin Microbiol 32:* 3018-3025.

Yang ZH, Mtoni I, Chonde M, Mwasekaga M, Fuursted K, Askgard DS, Bennedsen J, de Haas PEW, van Soolingen D, van Embden JDA, Andersen AB 1995. DNA fingerprinting and phenotyping of *Mycobacterium tuberculoiss* isolates from human immunodeficiency virus (HIV)-seropositive and HIV-seronegative patients in Tanzania. *J Clin Microbiol 33:* 1064-1069.

Yang ZH, Rendon A, Flores A, Medina R, Ijaz K, Flaca J, Eisenach KD, Bates JH, Villareal A, Cave MD 2001. A clinic based molecular epidemiological study of tuberculosis in Monterrey, Mexico. *Int J Tuber Lung Dis 5*: 313-320.

Yuen LKW, Ross BC, Jackson KM, Dwyer B 1993. Characterization *of Mycobacterium tuberculosis* strains from Vietnamese patients by southern blot hybridization. *J Clin Microbiol 31*: 1615-1618.