[a] *Instituto Nacional de Tecnología Agropecuaria, Centro Nacional de Investigaciones Agropecuarias, Instituto de Patobiología, Las Cabañas y los Reseros s/n, Casilla de Correo 25, 1712, Castelar, Provincia de Buenos Aires, Buenos Aires, Argentina*
[b] *Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, Junín 954, C1113AAD, Ciudad Autónoma de Buenos Aires, Buenos Aires, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Tecnológicas, Godoy Cruz 2290, C1425FQB, Cuidad Autónoma de Buenos Aires, Buenos Aires, Argentina*

[*] Corresponding author.
E-mail address: ggutkind@ffyb.uba.ar (G.O. Gutkind).

http://dx.doi.org/10.1016/j.ram.2017.02.001
0325-7541

Multiplex PCR assay for genotyping of *Mycobacterium tuberculosis* in Lima, Peru

PCR Múltiple para el Genotipaje de *Mycobacterium tuberculosis* en Lima, Perú

Dear Editor,

In South American countries it is necessary to have molecular methods as epidemiological tools as well as low costs and good level of discrimination capacity for polymorphisms that may enable molecular laboratories to perform the tasks for other regional laboratories having minimum standards. The methodology proposed in this article addresses this need.

We used the Proportion Method[2] for susceptibility testing of *Mycobacterium tuberculosis* isolates against isoniazid (INH), rifampicin (RMP), ethambutol (EMB), para-aminosalicylic acid (PAS), thioacetazone (T), kanamycin (KM) and streptomycin (SM). Cultures were grown on Lowenstein Jensen medium at 37 °C for 21 days, and slides were processed with the Ziehl–Neelsen stain. DNA from samples was extracted by using the phenol chloroform method[6]. In a simple PCR for *M. tuberculosis* strain differentiation, primer Mtb2 (5′-CGGCGGCAACGGCGGCA) was used with primers IS1 (5′-CGGACTCACCGGGGCGGTTCA) and IS2 (5′-CGGACATGCCGGGGCGGTTCA) that anneal at the inverted repeats flanking IS*6110*[7]. PCR was done in a mixture containing 25 pmol of each primer, 1 U of Platinum *Taq* DNA polymerase (Invitrogen), 0.2 mM of each deoxyribonucleotide triphosphate, 10 mM Tris–HCl (pH 8.4), 1.65 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton X-100 and overlaid with mineral oil. Cycling conditions were as follows: denaturation at 94 °C for 5 min, followed by amplification for 35 cycles of 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. A total of 20 μl of amplified DNA was subjected to electrophoresis in a 2% agarose gel, detected by ethidium bromide staining, and visualized under UV light. For the genetic polymorphism study, we used the Bionumerics program version 5.0 (Applied-Maths).

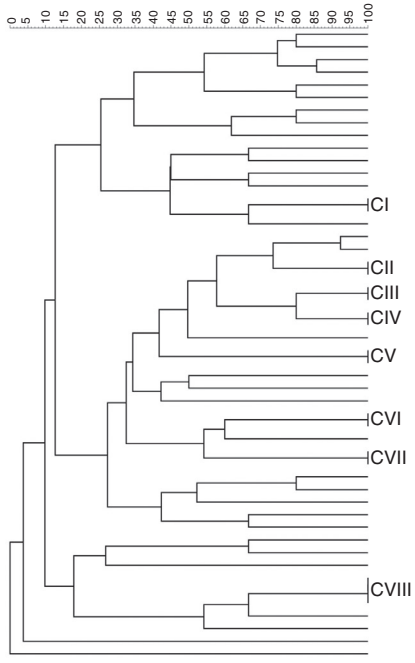
The drug resistance study showed that out of 49 strains belonging to TB patients in the Guillermo Almenara Irigoyen National Hospital, 18 strains (36.74%) were drug-sensitive, 10 (20.4%) were drug-resistant TB (DR) and 21 (42.8%) were multidrug-resistant TB (MDR), 3 of which were TB/HIV cases. Forty-two (42) different banding patterns were observed, which were classified into 10 clusters (Fig. 1). We suspect that cases MT009 and MT029 belong to heteroresistant strains, i.e. mixed wild-type and mutant strains because the banding pattern seems to overlap[5].

The transmission study showed Cluster II. Two male patients with MDR-TB, one of whom (MT014) had undergone previous MDR-TB treatment, were both hospitalized; Cluster IV. Two male patients with TB/HIV co-infection, both residing in the same district; Cluster VI. Two male patients with TB/HIV co-infection, both living in the same district and receiving their treatment in the same hospital, one of them (MT048) with DR TB and the other (MT054) with a sensitive case; Cluster VIII. Three male patients aged 44, 34 and 28 years, respectively, two of whom were brothers (MT033 and MT041) and the other a neighbor (MT032), all of them sensitive cases. The other clusters did not have an epidemiological link. A statistical risk study was performed[4,13] and the result was that the patients with HIV infections had the highest contagion risk in our population ($p = 0.174$; OR = 3.150; CI = 0.568–17.477). The repetition rate was good (Cronbach's alpha = 0.82). This genotyping method could be an alternative for other PCR-based typing procedures, such as spoligotyping and MIRU-VNTR typing as cited in other studies[7] and could help in the study of transmission relationship with heteroresistance, HIV-TB patients and outbreaks. Our TB survey system has many complications[3,9,11] and the lack of surveillance in DOTS[3] results in patients having a great diversity of genotypes and drug-resistant profiles[1,5], as well as heteroresistance of wild type to resistant, resistant to resistant, and wild type to MDR strains[1,4,8,10,12,13]. Our country needs a strategy based on epidemiology with molecular tools that will assist us in the analysis of the genetic diversity existing in Peru.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.



	Sex	Residence	Drug resistance detailed	Resistance	Other epidemiological dates
MT007	F	Callao	Sensitive	S	
MT011	F	Lima	Sensitive	S	
MT006	M	Callao	INH SM RMP EMB PAS T KM	MDR	
MT012	F	Lima	Sensitive	S	Health worker
MT005	M	Lima	Sensitive	S	
MT010	M	Lima	INH SM RMP EMB T KM	MDR	
MT003	M	Lima	Sensitive	S	HIV+
MT008	F	Lima	INH SM RMP PAS	MDR	HIV+
MT029	F	Lima	INH RMP KM	MDR	
MT026	M	Callao	SM	DR	
MT035	F	Callao	Sensitive	S	
MT024	M	Callao	INH SM RMP EMB	MDR	
MT028	F	Lima	INH SM RMP	MDR	
MT023	M	Lima	INH RMP EMB T	MDR	HIV+
MT025	M	Callao	Sensitive	S	Relapse
MT022	M	Lima	INH SM RMP EMB	MDR	Noncompliance
MT013	F		INH SM RMP	MDR	
MT016	M	Callao	INH SM RMP EMB PAS T KM	MDR	
MT014	M	Callao	INH RMP	MDR	Geriatric patient
MT015	M	Lima	INH SM RMP EMB T	MDR	Treatment failure
MT038	F	Callao	Sensitive	S	Relapse & failure
MT053	F	Lima	INH SM RMP EMB T	MDR	Failure
MT002	M	Callao	SM	DR	HIV+
MT040	M	Lima	INH SM RMP	MDR	HIV+
MT009	F	Callao	Sensitive	S	
MT043	F	Callao	Sensitive	S	Relapse & noncompliance
MT044	M	Lima	SM	DR	HIV+, noncompliance
MT051	M	Lima	SM RMP	DR	
MT055	M	Lima	INH SM RMP EMB T KM	MDR	
MT018	F	Callao	EMB T	DR	Health worker
MT048	M	Lima	INH SM T	DR	HIV+, treatment in the hospital
MT054	M	Lima	Sensitive	S	HIV+, treatment in the hospital
MT047	F	Lima	INH RMP EMB	MDR	
MT049	M	Callao	SM	DR	
MT050	F	Lima	INH SM RMP EMB PAS T KM	MDR	
MT017	M	Lima	SM RMP T	DR	
MT042	M	Lima	Sensitive	S	HIV+
MT020	F	Callao	Sensitive	S	
MT027	F	Callao	SM	DR	
MT052	F	Lima	INH SM RMP EMB	MDR	Pediatric
MT030	M	Lima	INH SM RMP EMB	MDR	Antituberculosis treatment
MT039	F	Lima	Sensitive	S	Health worker
MT000			Sensitive	S	H37Rv
MT032	M	Lima	Sensitive	S	Neighbour of MT041 & MT 033
MT033	M	Lima	Sensitive	S	Brother to MT041
MT041	M	Lima	Sensitive	S	Brother to MT033
MT021	M	Lima	INH SM RMP EMB T	MDR	
MT031	M	Lima	Sensitive	S	
MT034	F	Lima	INH RMP	MDR	
MT004	M	Lima	Sensitive	S	Pediatric

Figure 1 The dendogram of fragment to Multiplex PCR of 50 strains of *M. tuberculosis* was constructed using the similarity coefficient of Dice and UPGMA, using a tolerance of 1.2% and optimization of 0.17%. The *clusters* CI, CII, CIII, CIV, CV, CVI, CVII y CVIII are indicate in the dendogram. The banding patterns obtained by PCR-based methodology are in the right of the dendogram. The attached table contains demographic and drug resistant date of the cases.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Funding

Universidad Nacional Mayor de San Marcos: CON-CON 2006 – Con asignación a la Investigación y con incentivo al investigador.

Acknowledgments

The authors acknowledge to Dr. Pablo Ramirez Roca and Dra. Debora Alvarado Iparraguirre for the use of the Molecular Microbiology Laboratory (Universidad Nacional Mayor de San Marcos, Peru), Dr. Ernesto Montoro and Dr. Raul Diaz of Instituto Pedro Kouri (Cuba) for the advice and review of this investigation.

References

1. Baldeviano C, Quispe N, Bonilla C, Gastiaburu D, Pro J, Llanos-Zavalaga L. Perfiles genéticos (RFLP-IS6110) y resistencia a drogas en aislamientos de *M. tuberculosis* de pacientes internados en un hospital referencial del Callao, Perú. Rev Peru Med Exp Salud Publica. 2003;20:72–7.
2. Canetti G, Froman S, Grosset J, Hauduroy P, Langerova M, Mahler H, Meissner G, Mitchison D, Sula L. Mycobacteria: laboratory methods for testing drug sensitivity and resistance. Bull World Health Organ. 1963;29:565–78.
3. Del Castillo H, Mendoza-Ticona A, Saravia J, Somocurcio J. Epidemia de tuberculosis multidrogo resistente y extensivamente resistente a drogas (tbmdr/xdr) en el Perú: situación y propuestas para su control. Rev Peru Med Exp Salud Publica. 2009;26:380–6.
4. Foschiani I, Cardoso M, Araújo D, Suffys P. Drug-resistance and genotypes of strains of *Mycobacterium tuberculosis* isolated from human immunodeficiency virus infected and non-infected tuberculosis patients in Bauru, São Paulo, Brazil. Mem Inst Oswaldo Cruz. 2002;97:1147–52.
5. Gagneux S, Long C, Small P, Van T, Schoolnik G, Bohannan B. The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. Science. 2006;312:1944–6.
6. Hosek J, Svastova P, Moravkova M, Pavlik I, Bartos M. Methods of mycobacterial DNA isolation from different biological material: a review. Vet Med. 2006;51:180–92.
7. Kotlowski R, Chola Shamputa I, Abdullah El Aila N, Sajduda A, Rigouts L, van Deun A, Portaels F. PCR-based genotyping of *Mycobacterium tuberculosis* with new GC-rich repeated sequences and IS6110 inverted repeats used as primers. J Clin Microbiol. 2004;42:372–7.
8. Martiel J, Blot M. Transposable elements and fitness of bacteria. Theor Popul Biol. 2002;61:509–18.
9. Ministry of Health (Peru). Construyendo alianzas estratégicas para detener la tuberculosis: La experiencia peruana. Lima: MINSA/DGSP; 2006.
10. Miranda J, Ríos R, Clavijo A, Chacón C, Mattar S. Estudio preliminar de la susceptibilidad antimicrobiana y variabilidad genética de *Mycobacterium tuberculosis* en un área del Caribe colombiano. Coloma Med. 2006;37:275–86.
11. Pan-American Health Organization Tuberculosis in the Americas: regional report 2012. Washington, DC: Epidemiology, Control and Financing; 2013.
12. Pillay M, Sturm A. Evolution of the extensively drug-resistant F15/LAM4/KZN strain of mycobacterium tuberculosis in KwaZulu-Natal, South Africa. Clin Infect Dis. 2007;45:1409–14.
13. van Soolingen D, Borgdorff M, de Haas P, Sebek M, Veen J, Dessens M, Kremer K, Van Embden J. Molecular epidemiology of

tuberculosis in the Netherlands: a nationwide study from 1993 through 1997. J Infect Dis. 1999;180:726–36.

Carlos A. Tello[a,*], Nelly Borja[a], Ronnie G. Gavilan[b], Ruth Garcia-de-la-Guarda[c]

[a] *Hospital Guillermo Almenara Irigoyen, Lima, Peru*
[b] *Instituto Nacional de Salud, Lima, Peru*
[c] *Universidad Nacional Mayor de San Marcos, Lima, Peru*

* Corresponding author.
E-mail address: carolusmagnum@gmail.com (C.A. Tello).

http://dx.doi.org/10.1016/j.ram.2017.02.004
0325-7541