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Comparative molecular study of *Mycobacterium tuberculosis* strains, in times of antimicrobial drug resistance

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SUMMARY

Strains of *Mycobacterium tuberculosis* were compared using two DNA fingerprinting techniques: Restriction Fragment Length Polymorphism (RFLP) and Double-Repetitive-Element-PCR (DRE-PCR). Two of these strains: IH1 (susceptible to isoniazid) and IH2 (resistant to isoniazid) were recovered from cases of pulmonary tuberculosis which occurred in two brothers who lived together. The first one was recognized on July 1999, and the second was diagnosed one year later. IH1 and IH2 showed the same pattern of bands with both molecular tests. These results suggest that single drug chemoprophylaxis may occasionally select resistant strains for that drug, which can eventually cause disease and be recognized through these tests. Strains IH3, IH4 and IH5 were obtained from sputum samples of 3 different patients, and intra-laboratory cross-contamination was suspected when it was realized that the 3 positive materials had been consecutively processed the same day by the same worker in the same biological safety cabinet. Again, the 3 strains revealed identical band patterns with RFLP and DRE-PCR, confirming the posed suspicion. The results with DRE-PCR were obtained after only 8 hours of work, without the need for subcultures. This procedure allows quick correction of treatment conducts, avoiding unnecessary exposure of people and bacteria to antimicrobial drugs.

Key words: *Mycobacterium tuberculosis*, RFLP, DRE-PCR

RESUMEN

Estudio molecular comparativo de cepas de *Mycobacterium tuberculosis*, en tiempos de resistencia antimicrobiana a los fármacos. Se compararon cepas de *Mycobacterium tuberculosis* utilizando 2 procedimientos de ADN fingerprinting: polimorfismo de los fragmentos de restricción (RFLP) y Double-Repetitive-Element-PCR (DRE-PCR). Dos de las cepas: IH1 (susceptible a isoniazida) e IH2 (resistente a isoniazida) se recuperaron a partir de casos de tuberculosis pulmonar que ocurrieron en dos hermanos convivientes. La primera fue aislada en julio de 1999 y la segunda un año después. IH1 e IH2 mostraron el mismo patrón de bandas por ambos procedimientos. Estos resultados sugieren que la quimioprofilaxis con una sola droga puede ocasionalmente seleccionar mutantes resistentes, las cuales pueden causar enfermedad y ser reconocidas por estos procedimientos. Las cepas IH3, IH4 e IH5 fueron aisladas de 3 pacientes diferentes, y examinadas por probable contaminación cruzada dentro del laboratorio ya que fueron procesadas el mismo día, por el mismo operador y en la misma cabina de seguridad biológica. Nuevamente, las 3 cepas revelaron el mismo patrón de bandas por RFLP y por DRE-PCR, confirmando la sospecha. Los resultados de la DRE-PCR se obtuvieron luego de 8 horas de trabajo, sin necesidad de subcultivos. Esta técnica permitiría la rápida correción de pautas de tratamiento, evitando la exposición innecesaria de personas y bacterias a drogas antimicrobianas.

Palabras clave: *Mycobacterium tuberculosis*, RFLP, DRE-PCR

Several molecular techniques, jointly called "DNA fingerprinting" methods, that have been developed in the last decade, allow defining genetic relations or differences between microbial strains (1, 8, 10, 19).

Typing of cultures belonging to *Mycobacterium tuberculosis* bacterial complex through Restriction Fraction Length Polymorphism (RFLP) has shown to be clearly useful for studying tuberculosis outbreaks, identifying sources and chains of infection, and distinguishing reinfection from endogenous reactivation (2, 4, 12, 22, 23).

RFLP technique is based on the fact that different patterns of DNA fragments can be obtained in this analysis, depending upon the variable location of several copies of the insertion element IS*6110* in the bacterial chromosome. IS*6110* belongs to the IS*3* family of insertion segments, it can apparently act as a transposable fragment (10) distributed in the bacterial chromosome as 5 to 20 copies. The strains of *Mycobacterium tuberculosis* that show The strains of *Mycobacterium tuberculosis* that show identical RFLP pattern are considered to be clonally related.

It has been proven that the RFLP method is useful not only for studies of tuberculosis epidemiology, but also for deciding about possible cross-contamination in mycobacteriology laboratories (13).

While processing clinical samples (sputum, urine, etc) from a heavily infected patient, strains of *Mycobacterium tuberculosis* can be transferred to following negative samples. False positive cultures can thus originate, leading to erroneous diagnosis of tuberculosis and to long useless treatments. Fingerprinting techniques have demonstrated that these accidents are fairly frequent events in mycobacteriology laboratories.

Presently, the standard technique for comparison between *M. tuberculosis* strains is RFLP (21). However, this method has some drawbacks that limit its utility for situations like those we here report. Long processing times and heavy work required must be considered. An important amount of DNA is also needed, so further time-consuming subcultures may be necessary if primary cultures are scanty. Two weeks can elapse until meaningful results are obtained.

Alternative fingerprinting methods are being evaluated. Most of them are based on the amplification of hypervariable zones of the bacterial chromosome through Polymerase Chain Reaction (PCR).

DRE-PCR technique (double-repetitive-element) consists of the amplification of DNA segments situated between two copies of repetitive fragments: the above mentioned IS*6110*, or the Polymorphic Guanine-Cytosine Rich sequence (PGRS) (7). In *M. tuberculosis* genome, at least 30 copies of PGRS are included, their number and situation vary in different strains (17). DRE-PCR method is based on the fact that the number of copies and the distances between repetitive sequences IS*6110* and PGRS may differ among very similar strains.

DRE-PCR is quicker and requires fewer DNA copies than RFLP, so it can be often performed directly from the primary culture. Interpretable results can be obtained after 8 hours of work. This is not a minor fact, because when laboratory cross-contamination is suspected, confirmatory or discarding data should be readily produced (15).

The aim of the work we report in this paper was to compare *M. tuberculosis* strains through both abovementioned genetic procedures (DRE-PCR and RFLP) for studying two defined epidemiological situations. One of them consisted of the possible common origin of two infections caused by these bacteria, and the other situation referred to a presumed episode of laboratory cross-contamination resulting in undue treatment of patients.

Clinical and epidemiological records of patients were available from National Honorary Anti-Tuberculosis Commission, Uruguay. Primary microscopic and culture studies were done in its laboratory. Molecular studies were performed in Bacteriology and Virology Department, Institute of Hygiene, Medical School, Universidad de la República (State University, Uruguay).

Patients studied were:

- DGB, 21 years old, HIV negative. He had pulmonary tuberculosis diagnosed on July 14th 1999 through chest X ray examination, 3 positive smears (+++), and 2 positive cultures (+++) of *Mycobacterium tuberculosis* (IH1 strain) processed 15th and 17th July. Regular and supervised treatment was initiated 07/14/99 with isoniazid, rifampin and pyrazinamide during 3 months (3HRZ), the second stage of therapy was performed with isoniazid and rifampin twice weekly during 4 months (4H2R2). Cultures were negative after the second month of treatment, smears after fourth month. Clinical and radiologic evolution were good.

- HGB, 18 years old, HIV negative. He lived together with DGB, his brother, and had normal chest X ray controls. From 07/15/99 on he received chemoprophylaxis with isoniazid (300 mg/d) for three months. Clinical, paraclinical, and further X ray control exams performed every 3 months were normal.

On June 13th 2000, clinical and radiological findings led to a diagnosis of tuberculosis, that was confirmed with 2 positive direct smears of sputum (+++) and 2 positive *Mycobacterium tuberculosis* cultures processed 14 and 15th June 2000 (+++ confluent growth of IH2 strain, after 3 weeks incubation) Treatment schedule was the same as for DGB.

- AGC, 27 years old, HIV positive. Date of sample processing: 01/12/99. Negative direct smear, positive culture (+): 20-100 colonies of *Mycobacterium tuberculosis* (IH3 strain). Treatment was initiated with 3HRZ protocol, but changed to ER (ethambutol and rifampin) because susceptibility testing showed resistance to isoniazid.

- MC, 21 years, HIV negative. Three consecutive direct smears were negative, but sputum culture processed 01/12/99 was positive (5 colonies of *Mycobacterium tuberculosis*, IH4 strain). As for AGC, treatment was initiated with 3HRZ protocol, but was changed to ER because of isoniazid resistance.

- MF, 21 years, HIV positive. Three negative direct smears were obtained consecutively, but sputum culture performed 01/12/99 was positive (6 colonies of *M. tuberculosis*, IH5 strain). Therapy began with HREZ, but isoniazid was discontinued one month later due to drug resistance.

All strains of *Mycobacterium tuberculosis* were carefully examined in culture (Löwenstein-Jensen medium), microscopic smear stained with Ziehl-Neelsen method, niacin test and nitrate reduction assay (9). Susceptibility to antimicrobial agents was studied with the simplified variant of Canetti´s method of proportions (17). The cultures were kept at -70 °C in skim milk for further analysis.

For RFLP of *IS6110,* the bacteria were inoculated in Löwenstein-Jensen (L-J) medium and incubated at 37 °C until growth was visible.

DNA cleavage, separation of DNA restriction fragments by electrophoresis, Southern blot hybridization, and chemiluminescence detection were performed as described by van Embden et al (21). *Pvu*II-digested DNA was separated on a 0.8% agarose gel and hybridized with an IS*6110* probe

The probe was obtained by PCR using primers INS-1 (5´CGTGAGGGCATCGAGGTGGC3) and INS-2 (5´GC-GTAGGCGTCGGTGACAAA 3), that amplify an 245 bp DNA fragment extending from positions 889647 to 889891 of *M. tuberculosis* H37Rv. The amplified segment was marked with Amersham ECL kit for direct detection through chemiluminiscence.

For DRE-PCR, a loopful of bacteria grown on L-J medium was suspended in 1 ml distilled water, heated for 10 min. at 100 °C in a water bath, frozen overnight, defrost and centrifuged at 12000 rpm during 10 minutes. The supernatant was used as DNA template for amplification reactions.

The amplification mix was prepared with 5ul template DNA solution and 0,5 U *Taq* polymerase (Gibco BRL) in a total volume of 50 ul containing 50 mM Tris-HCl, 50 mM KCl (pH 8,8), 2,5mM MgCl2, 200uM dNTPs (Gibco BRL) and 0,5 pM primers.

Primers used were Ris1 (5´GGCTGAGGTCTCA-GATCAG), Ris2 (5´ACCCCATCCTTTCCAAGAAC), Pntb1 (5´CCGTTGCCGTACAGCTG) and Pntb2 (5´CCT-AGCCGAACCCTTTG).

The reaction protocol indicated 10 minutes initial dwell at 95 °C followed by 30 cycles of denaturing at 94 °C for 1 min, 2 min. annealing at 56 °C and 1 min. synthesis at 72 °C. GeneAmp® PCR system 2700 thermal cycler (Applied Biosystems) was used in all amplification reactions.

The amplification products were examined by 2% agarose gel electrophoresis.

Antimicrobial susceptibility tests were performed on all 5 strains examined, which were assayed against isoniacid, rifampin, streptomycin, ethambutol and pyrazinamide. Strain IH1 was susceptible to all drugs, but IH2 recovered from the epidemiologically related case and the other 3 cultures were resistant to isoniacid and susceptible to the other 4 drugs (Table 1).

IH1 and IH2 strains showed an identical pattern of bands both in RFLP (with 10 clearly defined bands, Fig. 1) and in DRE-PCR, that revealed amplified fragments of 200 to 1100 base pairs (Fig. 2).

Strains IH3, 4 and 5 yielded as well the same pattern of segments in RFLP (9 lines in total) and in DRE-PCR, which produced 8 bands from 100 to 600 bp approximately. These patterns were clearly different from those obtained with IH1 and IH2 cultures.

Considering the first situation examined, three hypothesis were posed to explain the infection detected in HGB one year after recognizing his brother´s illness: out-home contamination of HGB, contagion from his brother or common source infection.

IH1 and IH2 strains (obtained from DGB and HGB respectively) showed identical DNA band patterns in DRE-PCR and RFLP tests, supporting the last two hypothesis. Isoniazid resistance of IH2 attracted, though, our attention: both isolates were practically identical on a genetic basis, except for their susceptibility to isoniazid.

Table 1. Antimicrobial susceptibility of *M. tuberculosis* strains studied.

Strain	In vitro susceptibility to the following antimicrobial drugs[a]				
	INH	RMP	STM	EMB	PZA
IH1	S	S	S	S	S
IH2	R	S	S	S	S
IH3	R	S	S	S	S
IH4	R	S	S	S	S
IH5	R	S	S	S	S

[a] INH, isoniazid, RMP, rifampin, STM, streptomycin, EMB, ethambutol, PZA, pyrazinamide, S, susceptible, R, resistant.

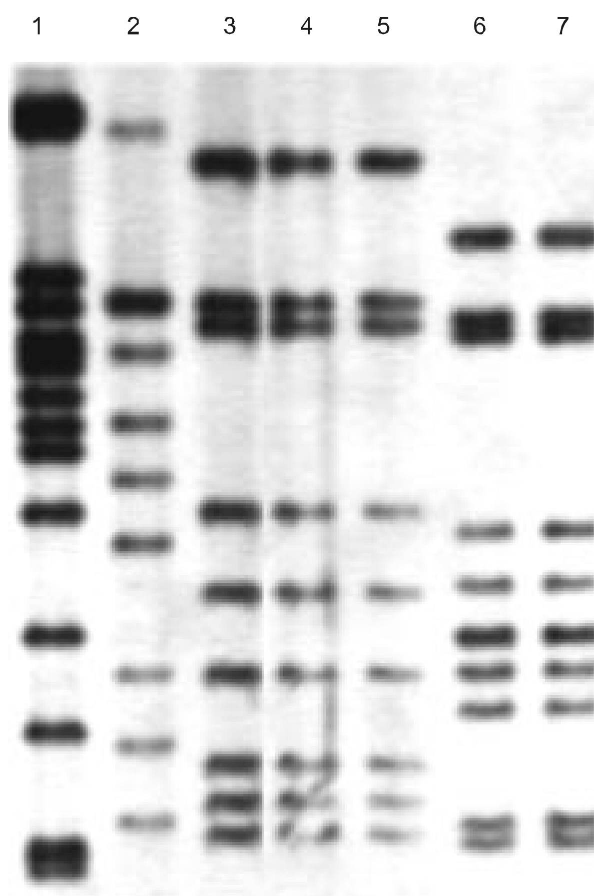

Figure 1. DRE-PCR applied to isolates of *M. tuberculosis*. Lane 1, IH3 strain, lane 2, IH4 strain, lane 3, IH5 strain, lane 4, *M. tuberculosis* H37Rv, lane 5, ladder 100 bp., lane 6, IH1 strain, lane 7, IH2 strain and lane 8, negative control.

A possible explanation was that the second patient may have been originally infected with a heavy bacterial burden, outweighing the probability of emergence of isoniazid resistant mutants. On these grounds, brief isoniazid prophylaxis may have acted merely as a limited monotherapy that favoured the selection of those mutants.

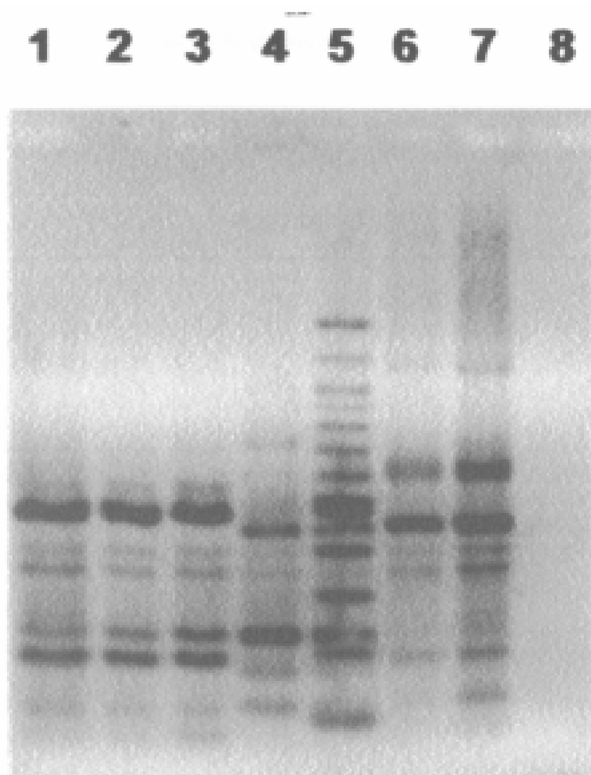

Figure 2. RFLP patterns of *M. tuberculosis* strains. Lane 1, *M. tuberculosis* H37Rv, lane 2, *M. tuberculosis* 14323, lane 3, IH3 strain, lane 4, IH4 strain, lane 5, IH5 strain, lane 6, IH1 strain and lane 7, IH2 strain.

Contrasting this explanation, it must be pointed out that clinical and radiological signs were absent during one year of regular follow up of HGB.

Single-drug chemoprophylaxis usage is biologically based on the assumption that the initial bacterial population of an infected person is not usually large enough to contain or produce a significant number of resistant mutants.

Further supporting this idea, an important clinical experience has shown that the evolution of patients in whom chemoprophylaxis failed was not clinically nor bacteriologically different from the evolution of infected individuals who developed disease after receiving placebo (6).

According to the results obtained with RFLP assay and DRE-PCR for IH1 and IH2 strains, it can though be proposed (in spite of the unexplained year-long asymptomatic period of HGB) that single drug chemoprophylaxis may sometimes select acquired resistance against the employed antimicrobial. This has been suggested by Ferebee (6) and Comstock et al (5), and reported by Toyota et al. after chemoprophylaxis performed in 1999 during a tuberculosis outbreak in a junior high school (20). In our present report we provide both detailed epidemiological and laboratory evidence to support this possibility.

Molecular techniques are being otherwise extensively used to study the selection and spread of antibiotic resistance in mycobacterial strains (11, 16, 18, 24).

The chances that HGB was contaminated with an already resistant strain coming from his brother are extremely low, because the regular and supervised combined therapeutic plan that DGB followed (3HRZ initial phase, 4H2R2 final phase) effectively prevents proliferation and diffusion of single-drug resistant bacteria.

The same described molecular techniques were separately applied to determine the extent of genetic relatedness among three strains of *Mycobacterium tuberculosis* isolated from respiratory tract samples of patients AGC, MC and MF, who were not epidemiologically linked. These samples were consecutively processed the same day by the same operator, in the same biosafety cabinet of the Mycobacteriology laboratory of the Honorary Anti-Tuberculosis Commission (CHLA). This laboratory is the national reference center for *Mycobacteria* (except *M. leprae*). It performs many primary microscopic studies and cultures of clinical samples sent from most health care facilities around the country, and also develops identification and susceptibility testing of the strains referred for study by some laboratories. 70-80 samples per day are analyzed, making an annual total of 25-26000, mostly (88%) sputum samples.

All tuberculosis cases in Uruguay must be notified to the National Commission, which works as an official institution dedicated to prevention, detection, treatment and epidemiological vigilance of tuberculosis in Uruguay. It produces and distributes BCG vaccine, and also keeps a complete record of cases and outbreaks, that are studied by inter-disciplinary teams of neumologists, social workers, epidemiologists and microbiologists. These characteristics of the institution and its laboratory explain both the need for precise controls and the availability of data examined in this report.

The three consecutive cultures analyzed (IH3, IH4 and IH5, respectively) were resistant to isoniazid and susceptible to all the other tested antimicrobials. They showed the same RFLP pattern and identical band distribution in DRE-PCR. These results strongly suggested that IH3, IH4 and IH5 were the same strain, and that cross-contamination had occurred in the laboratory, with AGC sample (bronchial lavage, +++ positive, 20-100 colonies on L-J medium) as the index material.

False positive cultures in diagnostic laboratories are the cause of long and unnecessary treatments. “Fingerprinting” techniques must be available for mycobacteriology laboratories (that deal with bacteria capable of tolerating harsh environmental conditions and even some disinfection procedures) to confirm or discard positive cultures suspected to be due to laboratory cross-contamination. When such a situation presents, results should be quickly produced, to avoid useless medication and unnecessary exposure of small populations of bacilli to resistance selective pressure. The major drawbacks of RFLP method to cope with this problem are the time required to obtain results through this procedure, and the complicated protocol

that must be followed. In the situation that we report, equally valuable results were obtained with DRE-PCR in an 8 hours total working time. We therefore believe that this method can be a good technical option in this type of situations, which appear in laboratories as CHLA that can process more than 100 samples per day, with a high risk of cross-contamination whose occurrence must be rapidly detected in order to guide therapeutic decisions.

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