

## Tuberculosis transmission of predominant genotypes of *Mycobacterium tuberculosis* in Northern suburbs of Buenos Aires city region

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### ABSTRACT

In 2003, the incidence of tuberculosis in Argentina showed an increase compared to 2002. The severe national crisis at the end of the 90s has probably strongly contributed to this situation. The goal of this work was to estimate the extent of the spread of the most predominant *Mycobacterium tuberculosis* strains and to assess the spread of predominant *M. tuberculosis* clusters as determined by spoligotyping and IS6110 RFLP. The study involved 590 pulmonary, smear-positive TB cases receiving medical attention at health centers and hospitals in Northern Buenos Aires (NBA) suburbs, from October 2001 to December 2002. From a total of 208 clinical isolates belonging to 6 major clusters, 63 (30.2%) isolates had identical spoligotyping and IS6110 RFLP pattern. Only 22.2% were shown to have epidemiological connections with another member of their respective cluster. In these major clusters, 30.2% of the 208 TB cases studied by both molecular techniques and contact tracing could be convincingly attributable to a recently acquired infection. This knowledge may be useful to assess the clonal distribution of predominant *M. tuberculosis* clusters in Argentina, which may make an impact on TB control strategies.

**Key words:** tuberculosis, clusters, predominant clones, genotyping

### RESUMEN

**Transmisión de la tuberculosis por genotipos predominantes de *Mycobacterium tuberculosis* en la región Gran Buenos Aires Norte.** La incidencia de la tuberculosis en Argentina mostró en 2003 un incremento en comparación con 2002. La grave crisis nacional a fines de los 90 ha probablemente contribuido en gran medida a esta situación. El objetivo del presente trabajo fue determinar la diversidad genética de aislamientos de *Mycobacterium tuberculosis* y el grado de dispersión de algunas cepas mayoritarias genéticamente relacionadas. El estudio involucró 590 aislamientos clínicos provenientes de muestras respiratorias con examen directo positivo, de pacientes atendidos en los hospitales y centros de salud que conforman la región Gran Buenos Aires Norte (NBA), de octubre de 2001 a diciembre de 2002. De 208 aislamientos que se encontraron en los 6 mayores *clusters*, 63 (30,2%) tenían patrones idénticos de *spoligotyping* y de IS6110 RFLP. En el 22,2% de los casos fue posible verificar la conexión epidemiológica con otro miembro del respectivo *cluster*. Concluimos que el 30,2% de estos agrupamientos principales pueden ser atribuidos a una infección reciente. Estos resultados pueden ser útiles para determinar la distribución clonal de los grupos predominantes de *M. tuberculosis* en Argentina, lo que puede impactar en las estrategias de control de la tuberculosis.

**Palabras clave:** tuberculosis, agrupamientos, clones predominantes, genotipificación

### INTRODUCTION

Tuberculosis (TB) incidence in Argentina has remained almost stable for the last 20 years. However, in 2003 the incidence was 32.0 per 100,000 inhabitants, showing a 2.5% increase in comparison with the year 2002 (30.5 per 100,000) (16). Furthermore, it varies significantly per area and high incidences are recorded in Buenos Aires Province (36.4 per 100,000), with the highest figures in the northern suburbs of Buenos Aires City (NBA, 46.0 per

100,000). During the year 2005, the incidence rate was 30.4 per 100,000 cases in Argentina, 33.2 per 100,000 in Buenos Aires Province and 43.2 per 100,000 in NBA. In this area, with more than 3.5 million inhabitants and 14 municipalities, a total of 1,480 new TB cases were reported in 2002 (16).

Molecular techniques for differentiation of *Mycobacterium tuberculosis* strains, such as restriction fragment length polymorphism (RFLP) and spoligotyping have been used for more than a decade to study the epidemiology

of TB (7, 8, 13, 18, 22, 28). RFLP analysis with IS6110 as a probe, was used in several epidemiological investigations, providing compelling evidence of institutional transmission (2, 3, 5, 23). Furthermore, this method was used to identify cases of unsuspected transmission, for tracking laboratory cross-contamination, and for discriminating exogenous versus endogenous infection (3, 4, 6, 20, 25). Besides, it is assumed that two or more clinical *M. tuberculosis* isolates sharing identical IS6110 RFLP patterns (defined as *cluster*) indicate in most cases both person-to-person transmission or transmission from a similar source, as well as a recently acquired infection (24, 29).

Spoligotyping, is also useful to differentiate the species belonging to the *M. tuberculosis* complex (12, 28). The ability of spoligotyping to differentiate *M. tuberculosis* strains is lower than that of IS6110 RFLP, but being a rapid and cheaper technique, it can be used to screen clinical isolates from a larger TB population (9, 11). Isolates with identical spoligotype patterns should be subjected to additional typing with the most discriminatory IS6110 RFLP to study tuberculosis transmission.

In a first approach, this study investigates the genetic diversity of predominant clinical isolates of *M. tuberculosis* from NBA patients by using spoligotyping and IS6110 RFLP. In addition, the aims were also to estimate the extent of spread of these predominant *M. tuberculosis* strains which form large TB clusters (larger than 10 patients).

## MATERIALS AND METHODS

### Population study

This study was carried out from October 2001 to December 2002, involving 622 cases from health institutions in the area NBA (21 health centres and 15 hospitals), which were requested to submit smears and/or cultures, as well as clinical and bacteriological data from the identified TB cases, to Dr. Cetrángolo Hospital. The information included location of the disease, previous treatment history, HIV infection, smears, cultures and drug susceptibility testing results. Epidemiological data obtained from the medical records were also included in the database: age; gender; residence; the fact of being a health care worker; household or occasional contact with a recent or past TB case. Contacts were identified by social workers interviewing the TB patient and more frequent contacts, followed by clinical radiological and if needed, bacteriological examination.

A total of 622 respiratory clinical specimens were processed by smears for acid-fast bacilli detection and cultures on Lowenstein-Jensen (LJ) medium and MGIT960 (BD, Buenos Aires, Argentina) (14, 31). The LCx *M. tuberculosis* Assay (Abbott Laboratories, USA) was used to identify the *M. tuberculosis* complex isolates.

The susceptibility to isoniazid (INH); streptomycin; rifampin (RMP) and ethambutol was investigated by the classical proportion method on LJ medium and MGIT960 (15). RMP-resistance was also explored by analysis of the *rhoB* gene of *M. tuberculosis* by using a reverse line blot hybridization assay following the previously published protocol (21).

Spoligotyping was carried out on all the isolates. Considering unlikely that isolates with different spoligopattern belong to the same cluster, IS6110 RFLP typing was performed on *M. tuberculosis* isolates that belonged to an spoligotyping cluster. From

previous studies and patients without any verifiable epidemiological relationship among them, we found different IS6110 RFLP patterns from isolates belonging to a small spoligotyping cluster (2 or 3 isolates) (data not published). Therefore, we decided to perform this last technique on clusters comprising at least 10 or more isolates. To estimate the highly predominating *M. tuberculosis* clones in the community, which could be responsible for recent transmission, IS6110 RFLP typing was carried out on all *M. tuberculosis* isolates that belonged to spoligotype clusters comprising 10 or more isolates. Both techniques were performed by following the standard protocols previously published (12, 27). Genetic patterns were analysed by the Gel Compar software (version 4.1, Applied Maths, St-Martens-Latem, Belgium). The IS6110 RFLP patterns were compared by using the Dice coefficient for calculating the similarities between the patterns, and by tolerating 1% position variation. Patients with genetically related isolates were considered clustered. Patients whose isolate patterns exactly matched were assigned a single cluster designation. For spoligotyping, a cluster was also defined as two or more strains with identical patterns.

The reference strain H37Rv was used as reference standard for drug-susceptibility testing as well as the molecular techniques used.

### Statistical methods

The MedCalc software (version 7.3.0.0, MedCalc®, Maria-kerke, Belgium) was used to calculate summary statistics of the included variables. The significance level was established at 5.0%. Univariate associations between demographic and clinical variables and the dependent variable (clustered isolates) were examined by frequency tables and the Chi square test. Associations were also examined by multiple logistic regression analysis.

## RESULTS

Data from 622 out of 803 (77.5%) respiratory TB cases notified to the NBA TBCP during this 14 month- period were collected. Neither epidemiological information nor clinical specimens from the remaining 181 cases were sent to the laboratory to be processed. Due to contaminated cultures 32 out of 622 (5.1%) cases were also excluded; 590 patients that represented 73.5% (590/803) of the bacteriologically proved individual cases were finally included in the study. The median age of the patients was 35.1 years (95% CI: 33.8-36.2. range: 1-78); 230 (39.0%) women and 360 (61.0%) men (P: 0.0001); 125 (21.2%) cases were co-infected with HIV; 115 (19.5%) cases had previously received TB treatment; 7 (1.2%) were health care workers, and in 48 (8.1%) cases, epidemiological links were demonstrated. The same demographic characteristics were investigated in the 213 respiratory TB cases not included in this study, which were notified to the NBA TBCP. This investigation demonstrated that the patient characteristics of this excluded group did not differ significantly from the 590 patients included in this study (data not shown).

Drug susceptibility testing showed that 434 out of 590 (73.6%) *M. tuberculosis* complex isolates were susceptible to first-line anti-tuberculosis drugs (MTS); 75 (12.7%) isolates were multidrug-resistant (MDR; resistant to at least INH and RMP), and 81 (13.7%) strains were resist-

ant to one or more drugs but not to isoniazid plus rifampicin (DR) (Table 1). This high occurrence of MDR strains was specific to this region and during the period studied, whereas the overall incidence in the country was lower. Besides, the MDR figures were obtained by including all the prevalent cases occurring in the NBA region. Furthermore, it does not represent the MDR incidence rate in NBA but the prevalence at that moment. Most of these cases had received previous anti-TB treatment.

All *M. tuberculosis* complex isolates were also subjected to analysis of the *rpoB* gene by the reverse line blot hybridisation assay. The assay correctly identified all RMP-resistant isolates and showed that three mutations conferring resistance to RMP predominated in the NBA population. These point mutations in the *rpoB* gene were: TCG Ser531Leu 74.7% (56/75); CAC His526 Asp 16.0% (12/75); and TGG Ser531Trp: 5.3% (4/75).

All 590 isolates were successfully subjected to spoligotyping as a screening technique, yielding 249 different spoligotype patterns; 190 out of 590 (32.2%) isolates showed a unique spoligotype pattern, whereas 67.8% (400/590) were grouped into 59 clusters with an average of 6.8 isolates (range 2-52) (Table 1). Table 1 shows the correlation between patient characteristics and

spoligotype results. No statistical differences were found between the patient characteristics whose isolates had unique patterns and those of patients clustered by spoligotyping, except that the comparison between children under 15 and adults aged 15-35 years neared statistical significance. Children aged under 15 years more frequently belonged to a spoligotype cluster than adults aged between 15 to 35 years: 22/26 (84.6%) versus 145/230 (63.1%) (P: 0.0002). Similarly, 250 (69.4%) men and 150 (65.2%) women were clustered (P: 0.3299). HIV-positive and HIV-negative cases were equally frequently found in spoligotype clusters 84 (67.2%) and 316 (68.0%), respectively (P: 0.9610), as cases were with (79; 68.7%) and without previous anti-TB treatment (321; 67.6%) (P: 0.9577). Table 1 shows that 58 (72.2%) DR, 287 (66.1%) MTS and 55 (74.3%) MDR organisms had clustered spoligotype patterns.

The isolates of the six largest spoligotype clusters, containing 13 to 52 isolates and involving 35.3% (208/590) of the strains, were subjected to IS6110 RFLP typing to investigate the extent to which large chains of transmission contribute to the tuberculosis problem in NBA. All six spoligotype clusters were sub-divided. Table 2 shows the heterogeneity among the IS6110 RFLP pat-

**Table 1.** Unique and clustered spoligotype patterns, drug resistance profile of the isolates and the epidemiologic and clinical characteristics of the studied patients.

SPO-pattern	Age (years)			Gender		HIV		Previous treatment		Drug resistance		
	≤15	>15/≤35	>35	F	M	P	N	Yes	No	MTS	MDR	DR
Cluster	22 <sup>(1)</sup> (84.6) <sup>(2)</sup>	145 (63.1)	233 (69.7)	150 (65.2)	250 (69.4)	84 (67.2)	316 (68.0)	79 (68.7)	321 (67.6)	287 (66.1)	55 (74.3)	58 (72.2)
Unique	4 (15.4)	85 (36.9)	101 (30.2)	80 (34.8)	110 (30.6)	41 (32.8)	149 (32.0)	36 (31.3)	154 (32.4)	147 (33.9)	20 (25.7)	23 (28.4)
Total	26 (4.4)	230 (39.0)	334 (56.6)	230 (39.0)	360 (61.0)	125 (21.2)	465 (78.8)	115 (19.5)	475 (80.5)	434 (73.6)	75 (12.7)	81 (13.7)

<sup>(1)</sup>Number and <sup>(2)</sup>percentage of spoligotype patterns

SPO: spoligotype; F: female; M: male; P: positive; N: negative; DR: drug-resistant strains; MTS: *Mycobacterium tuberculosis* fully drug-susceptible; MDR: multidrug-resistant strain.

**Table 2.** Percentage of similarity found among the IS6110 RFLP patterns from strains clustered by spoligotyping.

Cluster	Spoligotyping Octal code	N° isolates	Similarity degree (%) of IS6110 RFLP patterns					
			100	95	88	82	76	<62
A	77777777760771	52	6	32	6	8	-	-
B	77777607760771	50	15	-	-	16	12	7
C	776177607760771	50	16	4	5	8	16	1
D	77777777720771	27	7	10	4	-	3	3
E	00000004020771	16	13	2	1	-	-	-
F	777737607760770	13	6	4	3	-	-	-
<b>Total</b>		208	63	52	19	32	31	11

terns of the isolates clustered by spoligotyping, expressed as the percentage of similarity found among the RFLP patterns. Sixty-three out of 208 (30.2%) isolates had identical genetic profiles by both spoligotyping and IS6110 RFLP typing, representing six clusters of six to 16 isolates. A total of 115 (55.3%) out of these 208 isolates grouped in a spoligotype cluster, which contained isolates having more than 95% similarity in their RFLP patterns. Furthermore, 49.0% (102/208) of the strains identified by spoligotyping as belonging to a cluster shared between 95% and 80% similarity among their IS6110 RFLP banding patterns. Although the spoligotype pattern was identical, 20.2% (42/208) of the strains showed a similarity of only 76% to 62% among their IS6110 RFLP patterns (Table 2).

Figure 1 displays the prevalent IS6110 RFLP patterns related to the spoligotype clusters A, B, C, D, E and F. Two out of 7 isolates from health care workers were found in clusters: one of them belonged to the major cluster A and 4 were identified as MDR strains. One of these latter mentioned strains belonged to a cluster, which had caused the first MDR-TB nosocomial outbreak in our hospital from 1992 to 1994 (20).

Nosocomial and familiar transmission were verified among patients from cluster E and F respectively, whose isolates shared the same spoligotyping, IS6110 RFLP and drug-resistance patterns. Epidemiological connections among members of their respective cluster were found in 22.2% (14/63) of the clustered patients.

Nineteen out of 590 (3.2%) strains showed a spoligotype pattern characteristic of the Haarlem genotype (10, 18). This strain was previously identified as the causal agent that had provoked MDR-TB nosocomial outbreaks in Argentina (20, 23). From 6 patients, (1.0%) the obtained mycobacteria had spoligotyping and IS6110 RFLP patterns compatible with those of the Beijing family (17, 18). These organisms were isolated from a community living in a small district of our region.

Risk factors associated to recent transmission, expressed as strains clustered by IS6110 RFLP, were explored by logistic regression analysis. The results showed that an epidemiological connection (household or circumstantial contact with a confirmed TB case) was associated with clustering (O.R. 2.26, 95% CI: 0.96-5.32). When adjusted by gender, the association was even stronger in women than in men (O. R. 2.63, 95% CI: 0.79-8.82). HIV infection, MDR, and previous treatment showed a slight and similar association with clustering, but these associations were not significant; HIV, O. R. 1.06, 95% CI: 0.50-2.26; MDR-TB, O.R. 1.21, 95% CI: 0.55-2.67; and previously treated cases, O.R. 1.24, 95% CI: 0.57-2.69.

## DISCUSSION

This study has been conducted in a big area in the Northern suburbs of Buenos Aires City, attempting to assess the proportion of cases probably attributable to a recent transmission of *M. tuberculosis* predominant strains and the risk factors related to it.

One of the main goals was to provide information about predominant clones of *M. tuberculosis* actively causing TB disease in an Argentine community living in a well-delimited geographical area, as well as to estimate the amount of cases probably due to either reactivation of a latent infection or the acquisition of a new strain from an exogenous contagious source.

In this study, 35.2% (208/590) of the cases investigated by spoligotyping were grouped into 6 major clusters and from them, 30.2% (63/208) of the isolates analyzed by spoligotyping and IS6110 RFLP, were clustered by both methods. Besides, 22.2% (14/63) had verifiable epidemiologic connections with another member of their respective cluster.

From 6 patients (1.0%), the obtained mycobacteria had spoligotyping and IS6110 RFLP patterns compatible with

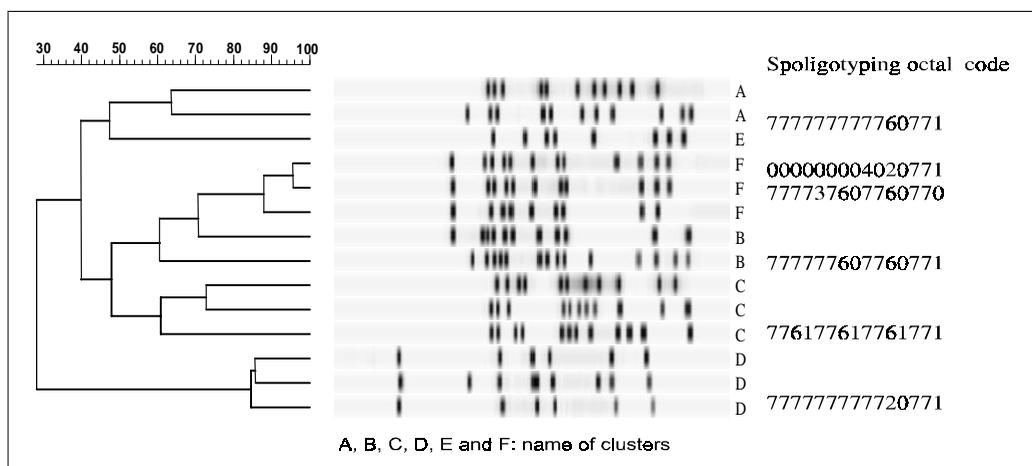


Figure 1. IS6110 RFLP patterns of the six predominant spoligotype clusters.

those of the Beijing family (17). These organisms were isolated from a community living in a small district of our region.

Clustering was more frequently associated to children and young people. Considering that the bacteriological diagnosis of TB in children is scarcely reached, the proved disease occurrence in them is an indicator of ongoing *M. tuberculosis* transmission in the community (2, 19). During this study period, isolates with identical spoligotyping and *IS6110* RFLP genetic patterns to that responsible for a MDR-TB nosocomial outbreak in the past and related to that of Haarlem strains (1, 20), were found to have caused active disease in 19 persons. Interestingly, these isolates considered to be a unique strain by genotyping, showed different drug-susceptibility patterns to anti-TB drugs and no epidemiologic links were found among these cases. These findings indicate that these isolates spread within the community.

No specific genetic patterns could be associated either to MDR or HIV cases.

In this study, spoligotyping correctly detected clustered cases confirmed by *IS6110* RFLP in the six major spoligotyping clusters. Taking into account that at least 30.2% of the cases in this study might be due to a recent transmission, the lack of specificity decreases the positive predictive value (56.10%) of spoligotyping. In addition to the fact that different spoligopatterns strains can be unlikely related, these findings reinforce the usefulness of spoligotyping as a screening technique.

Several factors might have contributed to the underestimation of the real amount of clustering in this community: losses of cases for technical reasons, the arbitrary selection of clusters with more than 10 isolates, and the assumption that a cluster should have been composed by strains with 100% of identity in their *IS6110* RFLP patterns (2). In that regard, several studies carried out on *IS6110* evolution, have shown that clonal variants that appear within a relatively short time and whose patterns differ by a few bands may represent recently evolved strains and therefore may be related to an ongoing transmission chain (19, 24, 26, 30).

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