

## Molecular Epidemiology of *Mycobacterium tuberculosis* Strains Isolated during a 3-Year Period (1993 to 1995) in Seville, Spain

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**The genetic polymorphism of *Mycobacterium tuberculosis* strains isolated in Seville, Spain, was studied by using computer-assisted analysis of the IS6110 fingerprint in order to determine the current situation and to evaluate the human-to-human transmission of this pathogen. One hundred seventy-six isolates from 175 patients among the 205 patients diagnosed with tuberculosis (TB) during a 3-year period (1993 to 1995) were cultured and analyzed. One hundred nine patients (62%) were infected with genetically different isolates, and 67 isolates (38%) were grouped into 19 clusters. These results demonstrate that the level of clustering of strains in Seville is intermediate between those in developed and developing countries. Epidemiological relatedness was shown for isolates from only 10 of these clusters. Active and high transmission rates exist in children and in human immunodeficiency virus (HIV)-infected adults, while in non-HIV-infected adults this transmission rate is moderate. Although transmission from children to adults is uncommon, the probability of transmission from HIV-infected patients to young adults not infected with HIV may be higher. On the basis of these observations, we predict a constant rise in the rate of TB transmission among HIV-infected patients and probably in young adult patients not infected with HIV if measures for the effective prevention of TB among the HIV-infected population are not implemented.**

The rates of tuberculosis (TB) reported from most developed countries have recently increased, thereby substantiating an earlier prediction of a 36% rise in the incidence of TB for the period between 1990 and 2000 (7). The resurgence of TB is the result of a number of factors, the most important of which is probably the increase in travel to and migration from areas of the world where TB is endemic and where there is a high incidence of TB. Other important factors include the present human immunodeficiency virus (HIV) pandemic and increasing levels of poverty in developed countries.

The typing of *Mycobacterium tuberculosis* strains is important for epidemiological control because it allows patients with TB to be linked and outbreaks to be traced to their sources with greater precision. It is also important to monitor species diversity and changing patterns of the disease. For example, typing of strains from all patients within a population will allow for an assessment of the relative importance of new infection, reinfection, and reactivation. It is hoped that conventional epidemiological principles and studies can be complemented by molecular methods so as to better elucidate the factors that promote the transmission of TB, which could lead to more efficient transmission prevention measures.

The most generally accepted molecular biology-based method for typing *M. tuberculosis* strains is restriction fragment length polymorphism (RFLP) analysis with the insertion sequence (IS) IS6110, which is used as a genetic marker of *M. tuberculosis* strain variability (27). This IS is present in multiple copies in *M. tuberculosis* strains (28), and the numbers and locations of IS elements within the chromosome vary between strains, resulting in a high degree of polymorphism (10). Despite the transposition capacity of this mobile genetic element (24), its stability has been studied extensively. No changes in the banding patterns of the *M. tuberculosis* strains have been detected

during passage in guinea pigs or during long-term, repeated serial culture in vitro. Sequential isolates, however, from chronic excretors or obtained during outbreaks of tuberculosis can yield one or two additional bands (6, 8, 14, 21, 25). For this reason, it is rational to assign isolates that differ by one band (a change reflecting a single genetic event) to the same cluster (23). Therefore, the analysis of IS6110-RFLP is a highly discriminatory and reproducible method useful for tracing sources during epidemics, distinguishing active transmission from reactivation, and detecting cross contamination during the processing of samples and strain dissemination (2, 9, 11, 12, 16, 20, 22, 25, 29).

Strains from various geographical regions have been typed by this method. Results demonstrate that in areas where the incidence of TB is low, there is a greater degree of polymorphism among the isolated strains. This occurs in most European countries (25, 28), where the majority of cases of TB result from the reactivation of lesions due to previously acquired infections. Conversely, in countries where the incidence is high, like in most African countries, many cases of TB are due to active transmission. This results in a low degree of polymorphism among the isolated strains (11, 29).

Spain has one of the highest incidence rates of both HIV infection and TB in Europe. The goal of the present study was to evaluate the genetic polymorphism of *M. tuberculosis* strains isolated from different population groups in Seville (during a 3-year study [1993 to 1995]), to assess the current situation, and to evaluate the human-to-human transmission of *M. tuberculosis* by comparing the epidemiological and clinical data with molecular typing data.

### MATERIALS AND METHODS

**Patients.** The study was performed at the University Hospital Virgen Macarena in Seville, Spain, an 850-bed general hospital that serves approximately 400,000 inhabitants and that contains 20% of the total public hospital beds in Seville. A total of 175 new patients admitted to the hospital between 1 January 1993 and 31 December 1995 with 176 episodes of bacteriologically confirmed TB were included in the study. Clinical data were collected for all patients. These data consisted of age, geographical origin, HIV serological status,

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clinical extension of the TB, and contacts with people with TB. A special epidemiological investigation was performed for every patient whose strain shared a fingerprint with a strain from another patient in the study. In such cases we conducted an active search for the transmission of TB through a study of the patient's history, contact with other patients in the study (if they were still alive), and the physicians who had treated the patient.

**Bacterial isolates.** One hundred ninety-six strains from 175 patients were studied. Clinical isolates were recovered from sputum ( $n = 131$ ), bronchoalveolar fluid ( $n = 6$ ), pleural fluid ( $n = 1$ ), gastric aspirates ( $n = 13$ ), lymph node aspirates ( $n = 9$ ), abscess aspirates ( $n = 5$ ), urine ( $n = 3$ ), cerebrospinal fluid ( $n = 4$ ), and feces ( $n = 4$ ). In cases in which more than one strain with identical RFLP patterns was obtained from the same patient, only one strain was considered. Only one patient was reinfected with a different strain during the study period.

**Bacteriological methods.** Direct smears prepared from clinical specimens were stained by the Ziehl-Neelsen (Difco, Detroit, Mich.) method. All specimens were digested and decontaminated by the *N*-acetyl-L-cysteine-NaOH (4% NaOH was used) method (13). The specimens were centrifuged at  $2,500 \times g$  for 15 min. The sediment was resuspended in 1 ml of 0.2% bovine serum albumin and was inoculated onto Löwenstein-Jensen slants with pyruvate (BioMedics, Barcelona, Spain) and 12B medium from the BACTEC system (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md.), a radiometric culture system. The cultures were incubated at 35°C and were observed for growth for up to 8 weeks. Commercially available probes for *M. tuberculosis* complex DNA (Accu-Probe; Gen-Probe, San Diego, Calif.) and standard biochemical tests (catalase, niacin production, and nitrate production) were used for identification of the isolates. The susceptibilities of the isolates to isoniazid, rifampin, streptomycin, ethambutol, and pyrazinamide were determined by the radiometric broth method (15). Isolates were maintained on Löwenstein-Jensen slants until assessment by RFLP analysis. Only one subculture was performed for each isolate.

**Fingerprinting by RFLP analysis.** With minor modifications (2), genomic extraction and Southern blotting were performed as described by van Embden et al. (27). Briefly, the DNA probe used in the hybridization was prepared by PCR with a labelling mixture with 2 mM (each) dATP, dCTP, and dGTP and 1.9 mM dTTP and with 0.1 mM digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) as the reporter molecule. The amplification product was examined by electrophoresis and was evaluated for the presence of a characteristic 245-bp product that results when the primers INS-1 (5'-CGTGAGGGCA TCGAGGTGGC) and INS-2 (5'-GCGTAGGCGTCGGTGACAAA) from IS6110 are used (27). The probe DNA was purified with a Sephadex column (Quick Spin Sephadex G-50; Pharmacia Biotech, Uppsala, Sweden).

Hybridization of the digoxigenin-labelled probe to the target DNA immobilized on nylon membranes was evaluated with a colorimetric system, the DIG Nucleic Acid Detection Kit (Boehringer Mannheim), by following the manufacturer's instructions. Genomic DNA from *M. tuberculosis* Mt14323, which gives 12 approximately evenly spaced bands of known sizes (17, 7.4, 7.1, 4.5, 3.6, 3.1, 2.1, 1.9, 1.7, 1.5, 1.4, and 1 kbp) when it is digested with *Pvu*II, was included in each gel as a reference standard.

**Computer analysis.** The computer-assisted analysis of the IS6110 fingerprints was performed by using GelCompar (Applied Maths, Kortrijk, Belgium). Imaging of Southern blots was performed with an HP ScanJet 4c scanner (Hewlett-Packard, Camas, Wash.) at 700 dots/in.<sup>2</sup>. Comparison of patterns was achieved by the unweighted pair group method with the arithmetic average clustering method and by using the Dice coefficient [(number of common bands  $\times$  2)/(number of bands in pattern A + number of bands in pattern B)]. If pattern A was identical to pattern B, a Dice coefficient of 0 was assigned. Conversely, if pattern A differed completely from pattern B, a Dice coefficient of 1 was assigned. Two bands were considered to be identical if they differed by  $\leq 3\%$  in their relative migration difference from the origin. The matrix of similarity shows the similarities between all pairs of patterns as squares shaded gray (the darker the square, the higher the correlation). A standardized residual  $\chi^2$  test with the Pearson correlation was used to perform univariate analysis.

**Definition of clustering.** A cluster of *M. tuberculosis* isolates was defined as two or more isolates which either exhibited the same number of copies (five or more) of the IS6110 fragment with identical molecular sizes or showed one additional or one missing IS6110 fragment. Clues to relatedness are considered to be formed by those strains with similarity coefficients of  $\geq 75\%$ .

## RESULTS

**Patients.** A total of 175 patients with 176 episodes of TB were studied. Forty-five strains were isolated in 1993, 67 strains were isolated in 1994, and 64 strains were isolated in 1995. Three groups of patients were established: children whose ages ranged from 1 to 14 years (14 strains), adults whose ages ranged from 15 to 49 years (127 strains), and adults older than 50 years (35 strains) (Table 1). Most patients (150 of 175 [86%]) presented with pulmonary TB, including 74 (42%) who had acid-fast-positive direct smears. Twenty-five other patients

TABLE 1. Isolates of *M. tuberculosis* by age and year of isolation

Age (yr)	No. of patients	No. of patients from whom strains were isolated in the following yr:		
		1993	1994	1995
$\leq 14$	14	4	8	2
15-49	127	30	47	50
$\geq 50$	35	11	12	12
Total	176	45	67	64

presented with extrapulmonary TB (14 patients with TB of the lymph nodes, 4 patients with meningitis, 3 patients with urinary tract infection, and 4 patients with gastrointestinal infection).

**Antibiotic susceptibilities of the *M. tuberculosis* isolates.** One hundred fifty-five (88%) of the 176 strains were susceptible to isoniazid, ethambutol, rifampin, pyrazinamide, and streptomycin. Twelve percent of the strains were resistant to at least one drug, with 10.7% of the strains resistant to isoniazid, 5.9% resistant to rifampin, 3.1% resistant to pyrazinamide, 0.7% resistant to streptomycin, and 0.3% resistant to ethambutol. Among these strains, 3.8% were multiple-drug-resistant (MDR) strains. When taking into consideration the year of isolation, 3.8% of MDR strains were found in 1993, 5.2% were found in 1994, and 2.6% were found in 1995. RFLP analysis of these resistant strains indicated no epidemiological relationship between them, supporting the observation that there had not been an outbreak involving MDR strains during these 3 years.

**IS6110 restriction fragment patterns.** The copy number of IS6110 in each of the isolates was determined from the number of bands hybridizing with the probe. The strains contained a mean number of  $9.5 \pm 3.0$  copies per isolate. One hundred sixty of the 176 strains (91%) contained between 5 and 14 copies of IS6110 (Fig. 1). These results are consistent with the 6 to 15 copies reported for isolates from other European countries (25, 28). None of the strains contained a single copy, as has been described for other populations (1, 9).

The strains were classified on the basis of computer-assisted comparison of DNA fragments, and a dendrogram showing the similarity between any two isolates or groups of isolates was constructed (Fig. 2A). One hundred twenty-eight distinct patterns were obtained from the 176 strains isolated (all the isolates from the same patient with identical RFLP patterns were considered only once). Nineteen banding patterns were shared

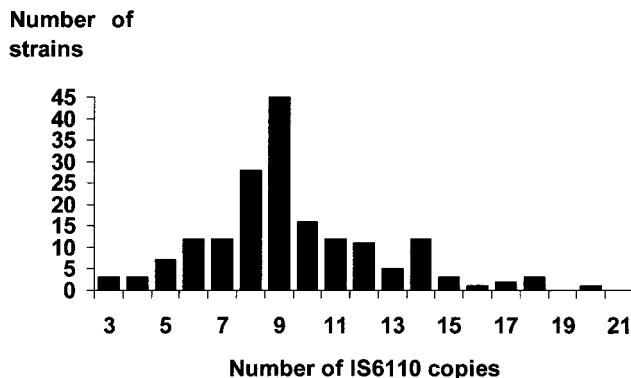


FIG. 1. Number of IS6110 copies in *M. tuberculosis* strains from 175 patients.

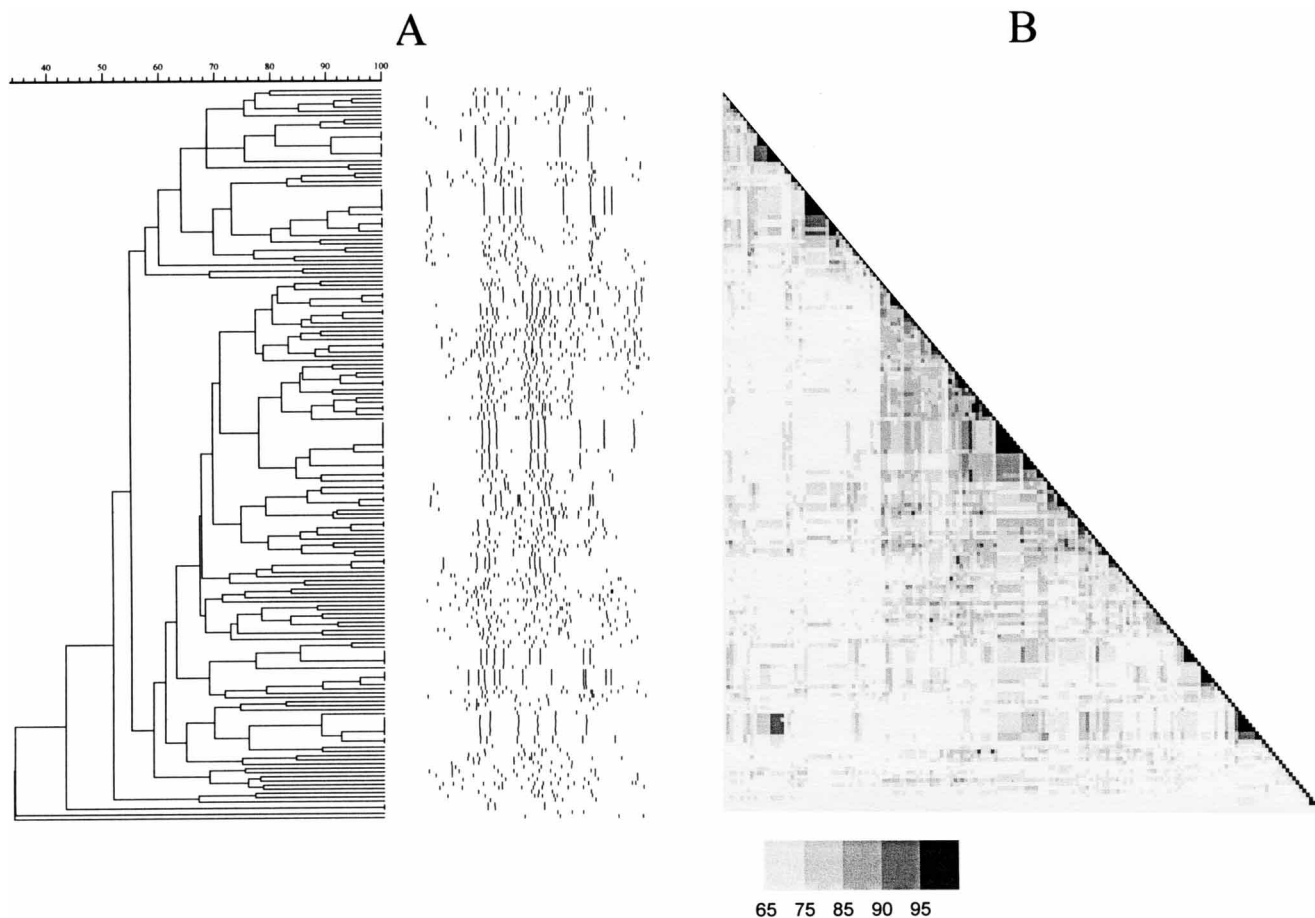


FIG. 2. IS6110 banding patterns and similarity matrix of 176 *M. tuberculosis* strains. (A) Dendrogram and banding patterns. The positions of the bands in each lane are adjusted (normalized) so that the band positions of all strains are mutually comparable and were determined by comparing the patterns with the IS6110 banding patterns of strain Mt14323, which was present in triplicate in each gel. The scale depicts similarity coefficients, as defined in Materials and Methods. (B) Similarity matrix. Similarity coefficients from 65 to 100% are shown by the five different gray tones in the matrix. The diagonal is formed by 100% similarity coefficient values of corresponding strains. Clues to relatedness are considered to be formed by strains with a similarity coefficient of  $\geq 75\%$ .

by two or more patients (Table 2). Sixty-seven strains (38%) belonging to these 19 clusters were found among the total of 176 strains, suggesting that the fraction of recently acquired infections in Seville was high. Clusters occurred in two to seven strains. Definite epidemiological relationships were clearly established in 10 clusters (Table 2). Four clusters (clusters B, E, F, and N) were found in HIV-positive inmates from the Seville II prison. Two clusters (clusters M and S; Fig. 3) corresponded to nosocomial transmission in pediatric wards. Clusters G and Q corresponded to an intrafamilial transmission, and clusters H and O corresponded to two intravenous drug user groups.

The other clusters could not be studied to determine the mechanism of transmission.

To visualize the relatedness between the banding patterns of all the isolates more objectively, a similarity matrix was generated (Fig. 2B). As can be seen in the similarity matrix, when the degree of relatedness of each IS6110 banding pattern with any other is studied, the degree of polymorphism among the strains is high and similar to that among strains isolated in other European countries (11, 25).

There were no differences between the strains infecting HIV-positive people and those strains isolated from the rest of

TABLE 2. Classification of *M. tuberculosis* strains by population type

Group	No. of strains (% of total strains)	No. (%) of strains in clusters	No. of strains in the following cluster:																		
			A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
Children (age, $\leq 14$ yr)	14 (8)	9 (64)								1	2			2				1		3	
HIV infected <sup>a</sup>	86 (49)	40 (47)		5	5	3	5	4		3		2	1		7	2	1	1	1		
Non-HIV infected <sup>a</sup>	41 (23)	13 (32)	2	1	3							2	1	1			1		1	1	
Age $\geq 50$ yr	35 (20)	5 (14)		1		1			1			1						1			
Total	176 (100)	67 (38)	2	7	8	4	5	4	2	3	2	3	2	2	3	7	2	2	3	2	4

<sup>a</sup> Ages ranged from 15 to 49 years.

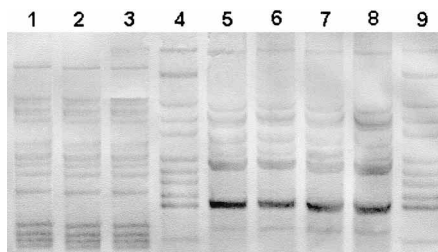


FIG. 3. Genomic fingerprints of *M. tuberculosis* isolates from two nosocomial outbreaks. Lanes 1 to 3, the three isolates from cluster M, respectively; lanes 4 and 9, reference strain *M. tuberculosis* Mt14323; lanes 5 to 8, the four isolates from cluster S, respectively.

the population studied. Similarly, there were no differences between the RFLP patterns for strains isolated from patients with extrapulmonary infection and those from patients with pulmonary TB.

**RFLP analysis and patient characteristics.** To analyze the transmission of TB, four groups of patients were studied (Table 2). The results indicated that the two groups with the greatest number of strains implicated in clusters were children (64%;  $P < 0.05$ ) and HIV-infected patients (46.5%;  $P < 0.05$ ), suggesting, as we expected, recent transmission of TB in these populations. Among the clustered strains isolated from children, five were implicated in two nosocomial outbreaks and one was implicated in an intrafamilial outbreak. Non-HIV-infected patients showed a lower number of strains in clusters (31.7%) than HIV-infected patients of similar ages, while in older patients the percentage of clustered strains was lower than expected (14%;  $P < 0.01$ ).

## DISCUSSION

During the last three decades, fiscal constraints have led to cutbacks in the Spanish TB control program. At the same time, the overlapping problems of HIV infection, homelessness, and substance abuse have caused a disproportionate increase in the number of TB infections, impeding the implementation of appropriate therapy and TB control measures in these groups. Incomplete treatment leads to continued TB transmission and an increased prevalence of drug-resistant *M. tuberculosis* strains. Due to all of these facts, the incidence of TB was considered to be near to 40/100,000 population (5) in 1992, but it continues to grow, especially in big cities (3).

Nevertheless, drug resistance rates did not increase during the period of study, being 13% among the strains isolated in 1993 (3.8% MDR strains), 14% among those isolated in 1994 (5.2% MDR strains), and 10% among those isolated in 1995 (2.6% MDR strains). This may be the result of better patient compliance with drug therapy and the change to a four-drug treatment regimen (4, 19, 29, 30).

The establishment of DNA fingerprinting techniques for *M. tuberculosis*, especially with the IS6110 probe, has made it possible to study the epidemiology of TB at the molecular level and to detect the sources of infection on the basis of the clonal differentiation of *M. tuberculosis* isolates. It is hoped that a better understanding of the dissemination of the bacteria in a defined population will improve the detection of new cases and the control of disease transmission. The present study was intended to provide an overview of the molecular epidemiology of TB in the region of Seville, which has a population of approximately 2 million, during the 3-year period from 1993 to 1995.

Analysis of the copy number of IS6110, with a mean of 9.5

and with strains containing between 5 and 14 copies, indicates that the *M. tuberculosis* strains in Seville are similar to those found in Zaragoza, Spain (17), and other regions of Europe (11, 18, 25, 26, 31). Our study revealed a total of 128 distinct RFLP patterns among the 176 strains analyzed. One hundred nine (62%) of the 176 strains infecting the 175 patients had unique fingerprints, and 67 strains (38%) were included in 1 of the 19 clusters detected. The matrix of similarity generated from the comparison of all the isolates (Fig. 2B) shows four clues of an indication (similarity coefficient,  $\geq 75\%$ ) but no clear-cut indication that the expansion of the clones, although related, is not of recent origin. These results suggest that the level of clustering of strains in Seville is intermediate between that in developed countries such as The Netherlands with 19% clustered strains (11) or France, with 28% clustered strains (26), and that in developing countries such as Tunisia, with 62% clustered strains (11), as well as other countries (28, 29).

Most cases of TB among patients older than age 50 years were due to reactivation, and the small number of strains from this group present in clusters (14%) made this patient group less important for the transmission of TB than the other groups studied. Although in non-HIV-infected adult patients there was a higher number of clustered strains, this population did not seem to play an important role in TB transmission.

This report has also demonstrated that two population groups in Seville have the highest percentages of clustered strains. If we assume that clustering is associated with recently acquired infection, children who are prone to the development of symptomatic TB, including severe disease, and HIV-infected patients, in which the progression of *M. tuberculosis* infection to active disease is often rapid, are the two groups of the population with the highest probability of showing recently acquired *M. tuberculosis* infection. All efforts at effective TB prevention and control and at reducing the TB rate in Seville should therefore concentrate on these groups, especially the HIV-infected population.

Finally, our study revealed three epidemiological groups: (i) children and HIV-infected people in whom an active and recent transmission of TB exists, (ii) people older than age 50 years suffering from the reactivation of a latent infection and with a very low incidence of active transmission, and (iii) non-HIV-infected adults in whom active transmission was moderate. Although transmission from children to adults is rare, the probability of transmission from HIV-infected patients to young adults not infected with HIV may be higher. Therefore, the situation in Seville predicts a constant rise in the rate of transmission of a limited number of prevalent *M. tuberculosis* strains among HIV-infected patients and probably among young adult patients not infected with HIV, as is the case in countries with a high incidence of TB (11, 29), if the efforts at effective TB prevention among the HIV-infected population are not taken. The relationship between both groups remains to be established and seems to be the most important factor in achieving real TB control in Seville.

This study will help to provide a better understanding of the TB situation in Spain and to compare it with that in other western European countries in order to assess the transmission patterns of TB in Europe. These data will also contribute to the global knowledge of TB transmission by describing geographical and demographic peculiarities, the nature of disease transmission among different populations, and the pathogenic potential of the strains and will help to achieve a better understanding of the impact of global migration, the HIV pandemic, and drug abuse on TB. When evaluated in global terms, the results described here may be used as guidelines for the

development of effective methods aimed at reducing the incidence and rate of transmission of TB.

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