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In Vivo IS*6110* Profile Changes in a *Mycobacterium tuberculosis* Strain as Determined by Tracking over 14 Years

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Transposition and homologous recombination of IS*6110* appear in *Mycobacterium tuberculosis* along *in vivo* sequential infections. These events were checked in different clones of a successful strain, *M. tuberculosis* Zaragoza, with the focus on a variant in which integration of a copy of IS*6110* in the origin of replication (*oriC*) region occurred.

Clonal *Mycobacterium tuberculosis* variants appear along the sequential infections of hosts involved in the same transmission cluster (1). IS*6110* in *M. tuberculosis* has been one of the most widely used elements in molecular epidemiology and has a transposition rate estimated at ~18% over a period of 5 to 6 years (2). The IS*6110* element itself modulates expression of neighboring genes and, depending on its location, could confer both transmission ability and virulence (3–5). Microevolution events due to the transposition of IS*6110* are usually reflected in variations of restriction fragment length polymorphism (RFLP) patterns (6, 7). Minor variations in IS*6110* fingerprints, spoligotypes, or mycobacterial interspersed repetitive-unit (MIRU)-variable-number tandem-repeat (VNTR) loci reveal microevolution in clonal infections (6, 8). These changes could have effects on the molecular structure of the mycobacterial genome and the biology of the bacillus (7).

Molecular epidemiological studies carried out for more than a decade have provided accuracy in the study of the transmission (9). The usefulness of the approach has been proven, even in countries with low incidences of tuberculosis (10). The *M. tuberculosis* Zaragoza strain spread in Zaragoza, Spain, and reached 18.7% of all isolates of *M. tuberculosis* in 2001 to 2004 (11). This strain was classified in principal genetic group 3 and in single-nucleotide polymorphism (SNP) cluster group 6a (12), it demonstrated the rare spoligotype SIT773, and it carried 12 localized copies of IS*6110* (13). In addition, in the context of a high-throughput survey of *in vivo* IS*6110* transposition in multiple *M. tuberculosis* genomes, an additional IS*6110* copy was detected in the *oriC* region in one isolate (14). The purpose of this study was to review the *in vivo* genomic changes of the *M. tuberculosis* Zaragoza strain, focusing on this variant.

Among the 2,348 isolates collected between 2000 and 2013, 246 were identified as *M. tuberculosis* Zaragoza by IS*6110* RFLP typing (15) and spoligotyping (16) in the context of tuberculosis surveillance in our region. By tracking the strain changes throughout time, we found 7 different variants, i.e., variants 1 to 7 (Fig. 1). An additional copy of IS*6110* was detected in six evolved clones, namely, variants 1 to 6. Variant 7 lost one copy in the RFLP pattern and was the only variant that revealed a change in its spoligotyping pattern. Clones 1, 2, 4, 5, 6, and 7 were isolated from only one patient, whereas variant 3 was isolated from 12 patients, as described below.

In variant 7, 10 of the 12 IS*6110* insertion sites known for

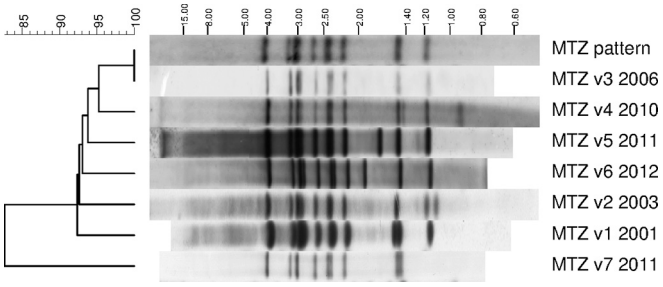

FIG 1 Dendrogram showing the IS*6110* RFLP patterns of one *M. tuberculosis* Zaragoza (MTZ) strain and its seven variants, including the years of isolation. The molecular sizes are indicated at the top (in kilobases).

M. tuberculosis Zaragoza were confirmed by PCR (13). However, the two nonamplified copies were located a relatively small distance from each other (9,860 bp for the H37Rv reference strain). The suspicion that homologous recombination between the two copies had occurred was corroborated by amplification of the expected sequence using the following external primers: DR43-F, 5′-ACCCGGTGCGATTCTGCG-3′; 2823-R, 5′-AAGGTGATCG AGGAGAAGTACCGGC-3′. The amplicon obtained in variant 7 (1,660 bp) was sequenced, and BLAST searching was performed (http://genolist.pasteur.fr/TubercuList). The sequence confirmed the deletion of ~10 kb, including the genes from *Rv2816c* to the point of insertion of IS*6110* in *Rv2823c*. The loss of this region is reflected in the lack of spacers 1 to 24 of the spoligotyping pattern (SIT585), classified as a Beijing-like pattern in the SITVITWEB database, in contrast to the pattern of the *M. tuberculosis* Zaragoza

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TABLE 1 Twenty-four-locus MIRU-VNTR patterns for five *M. tuberculosis* Zaragoza isolates, including three *M. tuberculosis* Zaragoza pattern isolates and two isolates of variant 3, showing the signature (2163b) detected from 2006

Isolate[a]	No. of repeats at position[b]:																							
	154	424	577	580	802	960	1644	1955	2059	2165	2347	2401	2461	2531	2687	2996	3007	3171	3192	3690	4052	4156	4348	2163b
MTZ-1	2	2	4	2	2	3	4	2	2	2	4	2	2	5	1	5	3	3	3	3	5	2	2	4
MTZ-2	2	2	4	2	2	3	4	2	2	2	4	2	2	5	1	5	3	3	3	3	5	2	2	4
MTZ-3	2	2	4	2	2	3	4	2	2	2	4	2	2	5	1	5	3	3	3	3	5	2	2	4
MTZ-v3-1	2	2	4	2	2	3	4	2	2	2	4	2	2	5	1	5	3	3	3	3	5	2	2	3
MTZ-v3-2	2	2	4	2	2	3	4	2	2	2	4	2	2	5	1	5	3	3	3	3	5	2	2	3

[a] MTZ, *M. tuberculosis* Zaragoza; MTZ-v3, *M. tuberculosis* Zaragoza variant 3.
[b] Loci are indicated by their positions (in kilobases) on the *M. tuberculosis* H37Rv chromosome.

strain (SIT773). This process of homologous recombination has been described to explain the deletion of other extensive regions (17). Our finding agrees with other studies indicating that strains with large numbers of IS*6110* copies have lost genomic regions due to recombination between copies (18). This mechanism was previously illustrated for other isolates of *M. tuberculosis*, demonstrating evolution of the spoligotyping pattern (19). Curiously, this microevolved clone did not spread to other patients.

In variant 3, a copy of IS*6110* was detected, in the context of a high-throughput survey of *in vivo* IS*6110* transposition, in the *oriC* region of the clone, which was not reflected in the RFLP pattern. Accordingly, it has been described that RFLP analysis can underestimate the real copy number for the IS*6110* element (14, 20). The o*riC* region in all available *M. tuberculosis* Zaragoza isolates was analyzed by PCR, which showed a fragment of 2,056 bp, reflecting a copy of IS*6110* in this region, for 12 isolates (5.06%) (21). The exact point of IS*6110* insertion (bp 1625 to 1627, with reference to H37Rv) and the direct orientation in the genome were confirmed by sequencing and gapped BLAST analysis. As a result of the IS*6110* transposition, 3-bp (CAC) direct repeats were generated.

The *oriC* region has been considered a hot spot in the genome of *M. tuberculosis*, as at least 10 different insertion sites for IS*6110* have been described (22). This location has been considered a specific marker to characterize the Beijing family (23, 24). The IS*6110* insertion point in variant 3 of *M. tuberculosis* Zaragoza is distinct from the points described, although it is relatively close (33 bp) to the Beijing point of insertion.

Some studies have suggested that the presence of one IS*6110* copy in the *oriC* region increases the duplication time for *M. tuberculosis* and this effect is independent of the orientation of the insertion element (25). It has also been reported that deletions in the *oriC* region can abolish the activity at the replication origin and that point mutations in the DnaA boxes severely reduce the activity of *oriC*. Concerning this, most IS*6110* copies have been located outside the DnaA boxes, and the *oriC* activity has not been affected (26). In this sense, the insertion of IS*6110* in the o*riC* region could be an evolutionary advantage (7). In our study, we observed that the insertion of IS*6110* is located outside the described DnaA boxes; therefore, we can assume that IS*6110* does not decrease the activity of the *oriC* region. Accordingly, and in contrast to the other variants, this variant was isolated from 12 patients.

A 24-locus MIRU-VNTR analysis (27) was performed for five *M. tuberculosis* Zaragoza isolates, and one difference in locus 2163b was observed in variant 3, with respect to the reference *M. tuberculosis* Zaragoza strain (Table 1). In order to study when this variation in the number of copies appeared, the 2163b VNTR was analyzed by simplex PCR amplification in all of the *M. tuberculosis* Zaragoza isolates. Nineteen of the isolates, including the 12 isolates of variant 3, showed 3 tandem repeats, in contrast to the rest of the isolates, which carried 4 copies. The fact that other isolates presented 3 copies of the repeated sequence in the 2163b locus in the absence of the IS*6110* insertion in *oriC* suggests that the event in 2163b occurred before the transposition of IS*6110* in the *oriC* region. Curiously, among the 7 isolates sharing this feature, two of the variants (variants 5 and 6) exhibited this variation, although no clinical significance could be attributed.

In conclusion, in monitoring of the *M. tuberculosis* Zaragoza strain for 14 years, different clones have been detected. Although the turnover of the markers used is not in the range of the pace of transmission, the insertion of an IS*6110* copy in *oriC* allowed us to distinguish one large subgroup. Variant 3 was able to be transmitted in a circumscribed area of the city. Interestingly, this well-studied outbreak will allow us to perform further research comparing variants belonging to the same isogenic group and therefore possessing the same genetic background. In addition, whole-genome sequencing would be useful for determining the microevolution of the *M. tuberculosis* Zaragoza strain during the well-documented outbreak and would be useful to validate the directionality and sequence of transmission (28).

The study and the protocols for collecting the bacterial strains were approved by the ethics committee of the Aragon government.

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