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Analysis of Mutations in Streptomycin-Resistant Strains Reveals a Simple and Reliable Genetic Marker for Identification of the *Mycobacterium tuberculosis* Beijing Genotype

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The *Mycobacterium tuberculosis* pandemic is a major health problem, further complicated by an increasing incidence of drug-resistant isolates and the existence of highly transmissible strains, such as those in the Beijing family. Streptomycin (STR)-resistant *M. tuberculosis* clinical isolates have been analyzed to look for mutations in the *rpsL*, *rrs*, and *gidB* genes. In addition, the *Rv1258c* gene, which encodes Tap, an efflux pump that transports STR, has been sequenced. Mutations affecting codons 43 and 88 of the *rpsL* gene were found in 44.4% of the strains, and 16.7% of the strains carried mutations in the *rrs* gene, both of which probably contribute to STR resistance. Many strains presented with mutations in the *gidB* gene, but the implication of those mutations in STR resistance remains unclear. Interestingly, a cytosine nucleotide insertion between positions 580 and 581 (denominated Tap^{580}) in the *Rv1258c* gene has been found in all Beijing isolates included in this study, suggesting that it might be a novel polymorphism specific to the Beijing family of *M. tuberculosis*. A simple and fast restriction fragment length polymorphism (RFLP)-PCR method for detecting the Tap^{580} insertion has been developed and used to screen a collection of 220 DNA samples obtained from cultures of *M. tuberculosis* isolates and 30 respiratory specimens. In all cases, the Beijing and non-Beijing representative samples were identified correctly. Tap^{580} is a novel polymorphism specific to the highly transmissible Beijing family, which allows for fast detection of these strains even at the very early stages of infection.

Mycobacterium tuberculosis is the cause of millions of incidences of tuberculosis infections and deaths worldwide (1), many of them among HIV-positive patients. This pandemic is further complicated by the increased incidence of drug-resistant strains (1), which generally have accumulated mutations in the genes that encode drug target or drug-activating enzymes. Nevertheless, there are resistant strains in which no mutations linked to drug resistance have been identified, hence suggesting unidentified drug-target proteins or novel mechanisms of drug resistance. A paradigm is the case of streptomycin (STR), an antituberculosis drug that is increasingly used due to the rising incidence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains. Mutations in the genes *rpsL*, *rrs*, and *gidB* (encoding the S12 ribosomal protein, 16S rRNA, and ribosome methyltransferase, respectively) have been found in ca. 70% of *M. tuberculosis* isolates that are resistant to STR; in the rest of STR-resistant *M. tuberculosis* strains, the cause of drug resistance remains unknown.

Molecular techniques have allowed for the differentiation of several lineages and families among *M. tuberculosis* strains, which differ not only in their global geographical distributions, but also in terms of transmissibility, drug resistance, treatment outcome, and other factors. The isolates of the Beijing family of *M. tuberculosis* are characterized by their increased ability to spread and cause disease and their increased association with drug resistance in comparison with the levels for non-Beijing strains (2). Because of this, many methods have been developed that are aimed at identifying and differentiating *M. tuberculosis* Beijing isolates (3–6). There are several methods for identifying Beijing isolates, including spoligotyping, restriction fragment length polymorphism (RFLP) using IS*6110* as a probe, or the presence of a copy of IS*6110* in the *dnaA* region (7). Other methods are based on the detection of specific genomic deletions, such as region of difference 105 (RD105) or an intact *pks15/1* gene (8). Recently, the detection of a single-nucleotide polymorphism (SNP) in the *Rv2629* gene by using real-time PCR followed by high-resolution melting has also been described (3).

In this work, we present an analysis of target mutations in clinical isolates of *M. tuberculosis* that are resistant to STR. In addition, the nucleotide sequence of the *Rv1258c* gene has been investigated as a potential STR-resistance determinant, since this gene encodes the drug efflux pump Tap in *M. tuberculosis*, which transports STR and other antibiotics (9–11). A nucleotide insertion in the coding sequence of the *Rv1258c* gene has been identified, which does not seem to be associated with resistance to this drug. Interestingly, this insertion is exclusive to the Beijing family of *M. tuberculosis* isolates and has led to the development of a method for screening *M. tuberculosis* strains (using either purified

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TABLE 1 Number of strains belonging to different lineages used in Tap^{580} screening

Lineage/species[a]	No. of strains
Beijing	49
T	27
LAM	25
Haarlem	18
U	16
CAS	12
EAI	9
S	2
X	1
Not assigned	48
M. africanum	13
Total	220

[a] LAM, Latin American-Mediterranean lineage; CAS, central Asian lineage; EAI, east African-Indian lineage.

DNA or directly on respiratory samples) and reliably identifying those belonging to the Beijing family of strains.

MATERIALS AND METHODS

***M. tuberculosis* DNA samples.** First, DNA samples of 18 *M. tuberculosis* clinical isolates that are resistant to STR and additional drugs (including several MDR isolates), of the Universidad de Zaragoza culture collection, along with the control strain H37Rv (which is fully drug susceptible) were used as a template to amplify and sequence the target genes (*rpsL*, *rrs*, *gidB*, and *Rv1258c*). This group included 4 strains that had a spoligotype that is consistent with being in the Beijing family.

Next, 220 DNA samples from *M. tuberculosis* complex clinical isolates were analyzed, some of them collected recently from hospitals in Huesca, Zaragoza, Madrid, and Barcelona (Spain), and some others from a Spanish national survey on MDR *M. tuberculosis* that was done between 1998 and 2009. This set included 49 DNA samples having a spoligotype that is consistent with those of the Beijing family, 158 that were representative of other *M. tuberculosis* distinct lineages (Latin American-Mediterranean [LAM], T, X, S, Haarlem, central Asian [CAS], east African-Indian [EAI], and others) including 5 isolates of the MTZ strain, a highly transmissible *M. tuberculosis* strain that has caused major outbreaks in Zaragoza (12), and 13 that were *Mycobacterium africanum* (Table 1). Overall, this collection included samples that were representative of most of the *M. tuberculosis* genetic lineages that have been described (13). In these samples, the presence of the novel nucleotide insertion in the *Rv1258c* gene that is described in this work was investigated. In a selection of 30 DNA samples (18 from Hospital Germans Trías i Pujol, Badalona, and 12 from Hospital General Universitario Gregorio Marañón, Madrid) that originated from STR-resistant isolates (five of them belong to the Beijing family), the *Rv1258c* gene was completely sequenced in order to verify the presence of the nucleotide insertion in the *Rv1258c* gene that is described in this work, and to identify other potential mutations.

PCR amplification and sequencing. The primers used for amplifying and sequencing the genes related to STR resistance, the annealing temperature used in the PCR, and the size of the product are listed in Table 2. PCRs were performed in a final volume of 50 μl using 200 μM (each) deoxynucleoside triphosphate (dNTP), 5 μl of buffer (10× PCR buffer; Applied Biosystems), 1.25 U of AmpliTaq Gold polymerase (Applied Biosystems), 0.25 μM (each) primer, 5 μl of dimethyl sulfoxide, and 2 μl of DNA (20 to 100 ng/μl). Amplifications consisted of an initial step at 94°C for 10 min, followed by 40 cycles of 1 min at 94°C, annealing for 2 min at the temperatures indicated in Table 2, and extension for 2 min at 72°C, with a final extension step of 10 min at 72°C. All genes were amplified in a single product, except for the *Rv1258c* gene, which was amplified in two overlapping products, one containing the promoter and the first half of the coding sequence, and the second containing the rest of the gene.

PCR products were purified using ExoSAP-IT (Affymetrix) and sequenced using the same primers used for the amplification. Sequences were then analyzed by comparison with that of the reference H37Rv strain (Cole et al. [14]; TubercuList database [http://tuberculist.epfl.ch/]).

Screening of nucleotide insertion in *Rv1258c* gene. First, an internal fragment of the *Rv1258c* gene containing the location of the cytosine nucleotide insertion between positions 580 and 581 of the coding sequence of the *Rv1258c* gene (denominated Tap^{580}) was amplified using the primers ctap9 and ctap10 (Table 2). DNA samples were processed for 9 min at 94°C, followed by 35 cycles of 30 s at 94°C, annealing for 30 s at 65°C, extension at 72°C for 90 s, and final extension at 72°C for 10 min. A product of 1,052 bp was obtained, which was digested for 1 h at 37°C with XhoI restriction enzyme, followed by electrophoresis in an 0.8% agarose gel in Tris-borate-EDTA (TBE) buffer. As a control of endonuclease digestion, PCR products were digested for 1 h at 37°C with PvuII restriction enzyme, which produces two fragments of 610 and 442 nucleotides for both Beijing and non-Beijing isolates.

Bioinformatic analysis. Databases of bacterial genomes were analyzed using the program BLAST at NCBI (National Center for Biotechnology Information, http://blast.ncbi.nlm.nih.gov/) to search for a Tap^{580}

TABLE 2 Primers used for amplification in this study

Target	PCR product (bp)	Ta[a] (°C)	Primer	Sequence (5′ → 3′)	Reference
For amplifying and sequencing					
rpsL	501	52	S13	GGCCGACAAACAGAACGT	30
			S16	GTTCACCAACTGGGTGAC	30
rrs	1,037	58	264	TGCACACAGGCCACAAGGGA	31
			285	GAGAGTTTGATCCTGGCTCAG	31
gidB	977	56	gidB3	GAACGGAAGATCGTCCAC	This work
			gidB4	CGATAGTTGAAGCCTGGC	This work
Rv1258c[b]	830	58	ctap1	CAATGTGGATTACCGCGAC	This work
			ctap2	GTCTTGCCGGTAGCCGTC	This work
Rv1258c[c]	902	58	ctap3	CGCAGGTTCCAGACGAAG	This work
			ctap4	GATCAGCGCGTTGAGTTC	This work
For detection of nucleotide insertion					
Rv1258c	1,052	65	ctap9	GTTGTTCGCCACGCTGGTCG	This work
			ctap10	CCAGATCCAGTTCGCGCAG	This work

[a] Ta, annealing temperature.
[b] 3′ region of the *Rv1258c* gene.
[c] Promoter and 5′ region of the *Rv1258c* gene.

TABLE 3 Mutations found in the *rpsL*, *rrs*, *gidB*, and *Rv1258c* genes in strains of the Universidad de Zaragoza culture collection

M. tuberculosis STR-resistant strain	Mutation found in:						
	rpsL			*gidB*		*Rv1258c*	
	DNA	Protein	*rrs* DNA	DNA	Protein	DNA[c]	Protein
Z-07044			C340T				
Z-07047[a]	A128G	K43R		A276C[b]	E92D	InsC 580–581	194 frameshift
				A615G[b]	Silent		
HMS-1838				C413T[b]	A138V		
HCU-2879	A263G	K88R					
HCU-2934	A128G	K43R		T47G[b]	L16R		
HCU-2830[a]	A128G	K43R		A276C[b]	E92D	InsC 580–581	194 frameshift
				A615G[b]	Silent		
HMS-1695	A128G	K43R	C492T	T47G[b]	L16R		
HMS-1691	A263G	K88R				A13del	5 frameshift
HMS-1781				G490C	G164R		
VEN-4145[a]	A128G	K43R		A276C[b]	E92D	InsC 580–581	194 frameshift
				A615G[b]	Silent		
VEN-1714				T47G[b]	L16R		
				C409G[b]	R137G		
VEN-5292			A324G	T47G[b]	L16R		
				T149C	L50P		
VEN-2457				T47G[b]	L16R		
VEN-1667				T47G[b]	L16R		
				G248C	R83P		
VEN-2543							
VEN-314				C159T	Silent		
VEN-4237			C462T	C159T	Silent		
			A736G				
VEN-3748[a]	A128G	K43R		A276C[b]	E92D	InsC 580–581	194 frameshift
				A615G[b]	Silent		

[a] Strains belonging to the Beijing family.
[b] Mutations found in susceptible strains (20, 21, 23).
[c] "InsC 580-581" indicates insertion of a C nucleotide between nucleotides 580 and 581. "A13del" indicates deletion of the A nucleotide at position 13.

insertion in the *Rv1258c* gene among the sequenced genomes of the *M. tuberculosis* complex.

Screening of Tap^{580} insertion in *Rv1258c* gene directly in clinical samples. A blind panel of 30 respiratory samples was assembled by two hospitals (Servicio de Microbiología, Hospital Universitario Lozano Blesa, Zaragoza, Spain, and Servicio de Microbiología Clínica y Enfermedades Infecciosas, Hospital General Universitario Gregorio Marañón, Madrid, Spain). This panel included 29 smear-positive and culture-positive samples from patients diagnosed with tuberculosis caused by Beijing (2 samples) and non-Beijing (27 samples) strains, and one further sputum sample that contained *Mycobacterium fortuitum* as a negative control. Of the samples containing *M. tuberculosis*, nine contained between 2 and 10 acid-fast bacilli/field, five samples contained between 10 and 100 acid-fast bacilli/field, and 15 samples contained >100 acid-fast bacilli/field. All samples were decontaminated by *N*-acetyl-L-cysteine–NaOH, and a fraction was incubated for 20 min at 80°C to kill the live bacilli and extract DNA using the GenoLyse kit (Hain Lifescience). The DNA samples were processed according to the method described above for detecting the Tap^{580} insertion in the *Rv1258c* gene.

RESULTS AND DISCUSSION

Mutations in *rpsL*, *rrs*, and *gidB* genes. In order to find the mutations responsible for STR resistance in 18 *M. tuberculosis* clinical strains, the genes *rpsL*, *rrs*, and *gidB*, which are known to carry mutations related to resistance to STR, were sequenced and compared with the sequence of the STR-susceptible H37Rv laboratory strain available in the TubercuList database (http://tuberculist.epfl.ch/). All strains analyzed in our study, including the reference H37Rv strain, had the mutations A363G in *rpsL* and C299T in *gidB*, which have been reported as sequencing errors in the sequence of H37Rv included in both the TubercuList database and GenBank accession no. AL123456.2 (15, 16).

The significant mutations found in the strains analyzed in our study are summarized in Table 3.

Eight of the strains tested (44.4%) carried mutations in the *rpsL* gene, A128G being the mutation that was found in six of them. The four Beijing strains included in this group carried this mutation, which is the most frequent mutation found in STR-resistant *M. tuberculosis* Beijing strains (17, 18). Two other strains (11.1%) had the mutation A263G.

Four strains (22.2%) harbored mutations in the *rrs* gene. Three strains carried the C340T, A324G, or A736G mutation; to our knowledge, this is the first report on mutations in this region of the *rrs* gene. The strain carrying the mutation A736G also carried the mutation C462T according to TubercuList numbering, a mutation that has been described as C461T in a previous publication (19). Finally, one strain carried the mutation C492T close to the 530 loop of the secondary structure of *M. tuberculosis* 16S rRNA; this strain is discussed further below.

The role of the GidB protein in conferring high-level STR resistance in *M. tuberculosis* has been fully characterized (20), although the contribution of certain point mutations in STR resistance is still controversial; it has been speculated that some mutations in the *gidB* gene might promote the acquisition of mu-

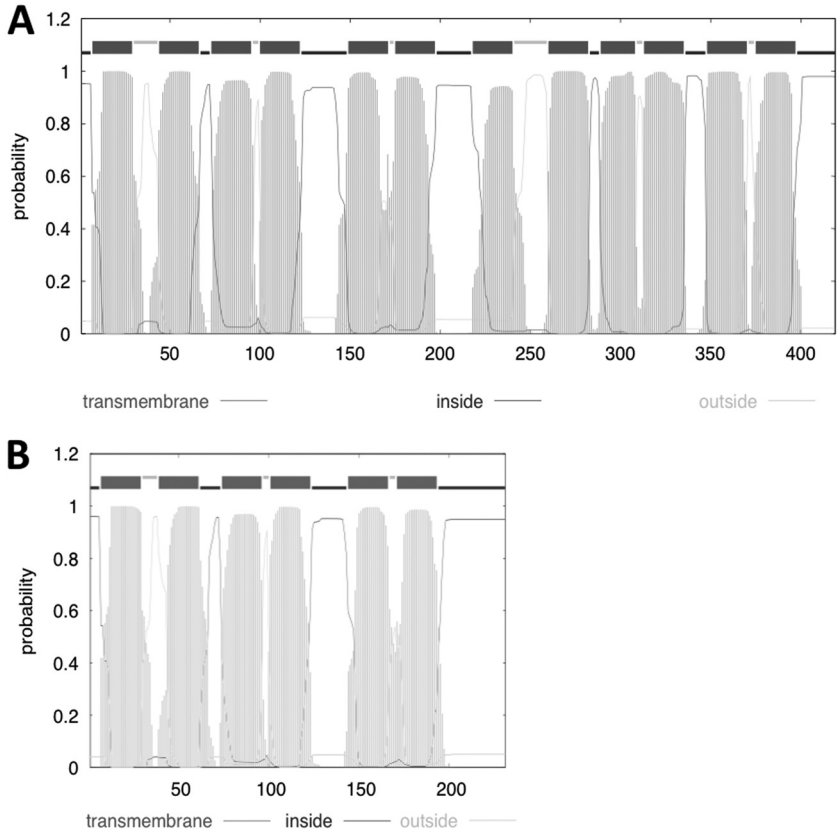

FIG 1 Hydrophobicity profile and transmembrane prediction of the Rv1258c proteins of *M. tuberculosis* H37Rv (A) and Beijing isolates (B) made by using the Hidden Markov model (TMHMM Server v2.0; Center for Biological Sequence Analysis, Technical University of Denmark [http://www.cbs.dtu.dk/services/TMHMM/]).

tations in either *rpsL* or *rrs* (21). A large number of mutations (including many silent mutations) were found in the *gidB* gene; in fact, only four strains had a *gidB* gene identical to that of H37Rv. Many missense mutations have been described in STR-susceptible strains, such as the mutation T47G (the most frequent mutation found in our study), which has been reported as being specific to the Latin American-Mediterranean (LAM) family strains (22); in fact, the 6 strains carrying this mutation in our study belong to the LAM family of strains (data not shown). The four Beijing strains carried two mutations: A276C (E92D), which has been described as an SNP specific to this family (22), and A615G; both mutations can be found in STR-susceptible strains (20). In the Beijing strains, these *gidB* mutations occur simultaneously with the mutation A128G in the *rpsL* gene, as mentioned above.

Among the non-Beijing strains, the *gidB* C413T mutation has been found in both STR-resistant and -susceptible *M. tuberculosis* isolates (20, 21, 23). One STR-resistant isolate carried the mutation G248C, indicating that it might lead to resistance to this drug; however, other mutations in this position (G248T) have been found in STR-susceptible strains (21). Finally, the only *gidB* mutation potentially related to resistance to STR is the missense mutation G490C, since this one was detected in a strain lacking mutations in the *rpsL* and *rrs* genes.

One strain carried mutations in all three genes; these were *rpsL* A128G, *rrs* C492T, and *gidB* T47G. In this strain, STR resistance is due to the mutation *rpsL* A128G, since the other two mutations have been found in both STR-resistant and -susceptible isolates. The mutation *rrs* C492T, also reported elsewhere as C491T, has been associated with the LAM3 genetic lineage of *M. tuberculosis* (24, 25). This very same genetic lineage of *M. tuberculosis* also carries the mutation T47G, as mentioned above (22).

Finally, one of the STR-resistant strains lacked mutations in any of the three genes *rpsL*, *rrs*, and *gidB*. In three other strains, only mutations in *gidB* were found, although these either were silent or have been described also in STR-susceptible strains, hence making it unlikely that they make a major contribution to resistance to this drug. This confirms that other mechanisms must contribute to STR resistance in *M. tuberculosis*.

Mutations in *Rv1258c* gene. Since STR is a substrate of the Rv1258c efflux pump (11), we hypothesize that mutations affecting the expression levels of the *Rv1258c* gene or that change the kinetic properties of the efflux pump might contribute to STR resistance. To investigate this further, the *Rv1258c* gene was amplified and sequenced in the 18 samples of *M. tuberculosis* STR-resistant clinical strains in which the *rpsL*, *rrs*, and *gidB* genes had been sequenced. Two different mutations in the *Rv1258c* gene were found. One strain has a deletion of the adenine nucleotide in position 13 (Table 3), producing a frameshift; as a result, a TGA stop codon ends translation of a peptide of only 10 amino acids. Four strains have an insertion of a cytosine nucleotide between positions 580 and 581 (Tap^{580}) (Table 3); this insertion causes a frameshift mutation from codon 194 onwards, resulting in a shorter protein (231 amino acids). This protein probably would not constitute a functional membrane transporter, since it contains only 6 transmembrane segments (TMS) compared with the 12 TMS of the full-length protein (419 amino acids) (Fig. 1). Bac-

TABLE 4 Search for Tap^{580} insertion by bioinformatics analysis of sequenced genomes of the *M. tuberculosis* complex

Strain	Beijing lineage	Tap^{580} insertion
Mycobacterium tuberculosis		
H37Ra	No	No
H37Rv	No	No
CCDC5079	Yes	Yes
CCDC5180	Yes	Yes
CDC1551	No	No
CTRI-2	No	No
F11	No	No
KZN 605	No	No
KZN 1435	No	No
KZN 4207	No	No
RGTB327	No	No
RGTB423	No	No
Strain Erdman = ATCC 35801 DNA	No	No
UT205	No	No
7199-99	No	No
Mycobacterium bovis		
BCG Pasteur 1173P2		No
BCG Mexico		No
BCG Moreau RDJ		No
BCG Tokyo 172 DNA		No
AF2122/97		No

terial drug efflux pumps of the major facilitator superfamily have 12 or 14 TMS, which are required for transport activity.

These five strains carrying mutations in the *Rv1258c* gene also carried the A263G (K88R) or A128G (K43R) mutation in the *rpsL* gene, which is probably responsible for the high-level STR resistance of these strains; then, it is difficult to analyze the contribution of *Rv1258c* mutations in resistance to this drug.

Remarkably, the four strains carrying the Tap^{580} insertion had been typed as belonging to the Beijing lineage by RFLP and/or spoligotyping (data not shown). We hypothesized that this might represent a novel polymorphism specific to the Beijing family. To test this, the presence of the Tap^{580} insertion was inspected in 15 genomes of *M. tuberculosis* available in public databases (NCBI), including clinical isolates, laboratory strains, such as H37Rv and H37Ra, and two strains of the Beijing family. Interestingly, only CCDC5079 and CCDC5081, which belong to the Beijing family (26), had the Tap^{580} insertion (Table 4), further supporting that Tap^{580} is an insertion that is specific to the Beijing family isolates. Other species of the *M. tuberculosis* complex, such as four substrains of *Mycobacterium bovis* BCG and *M. bovis* AF2122/97, did not show the Tap^{580} polymorphism and had complete identity with the *Rv1258c* gene of H37Rv (Table 4).

The Tap^{580} insertion is present in clinical strains of the Beijing family of isolates. The presence of the Tap^{580} insertion was further investigated in different subtypes of the Beijing family of isolates, and in isolates of other genetic families. For this, a quick and simple method for detecting the Tap^{580} insertion was developed. Between positions 577 and 582 of the *Rv1258c* gene, the sequence CTCGAG is the target for XhoI endonuclease; the insertion of a cytosine nucleotide in the Beijing strains results in CTC GCAG, which is not recognized by this endonuclease (Fig. 2). We designed two primers for amplifying a 1,052-bp fragment of the *Rv1258c* gene, from nucleotides 165 to 1216, which includes the position of the Tap^{580} insertion. Digestion of PCR products with XhoI endonuclease resulted in two DNA fragments of 413 and 639 bp in the case of non-Beijing strains, whereas PCR products from Beijing strains remained unaffected (Fig. 2).

Next, a collection of 220 clinical isolates of *M. tuberculosis* complex were screened, which included 49 isolates having a spoligotype that is consistent with that of the Beijing family. DNA samples were given random numbers and were blind tested using our RFLP-PCR method described above. The Tap^{580} insertion was present in all Beijing strains and absent in all strains belonging to other lineages of *M. tuberculosis*; the Tap^{580} insertion was also absent from *M. africanum* isolates (Table 1).

Two controls were included in our assays. First, in each experiment, samples of DNA from the laboratory strain *M. tuberculosis* H37Rv (which does not belong to the Beijing family of isolates) and from the *M. tuberculosis* GC1237 strain (a Beijing isolate recently characterized [27]) were included. The PCR product from *M. tuberculosis* H37Rv was cut by XhoI, whereas that of *M. tuberculosis* GC1237 was not cut, confirming that all steps in the identification process were carried out satisfactorily. Second, all PCR products were digested with PvuII restriction enzyme, which cuts the amplification products of the *Rv1258c* gene obtained from both Beijing and non-Beijing samples, demonstrating that the amplification step is specific to the *Rv1258c* gene and ruling out the possibility of unspecific amplifications.

Finally, out of the 220 samples, the *Rv1258c* gene was sequenced in the 30 *M. tuberculosis* STR-resistant clinical isolates from Hospital Gregorio Marañón (Madrid, Spain) and Hospital Germans Trias i Pujol (Badalona, Spain). The Tap^{580} insertion was consistently found in the 5 Beijing strains and was absent in all the

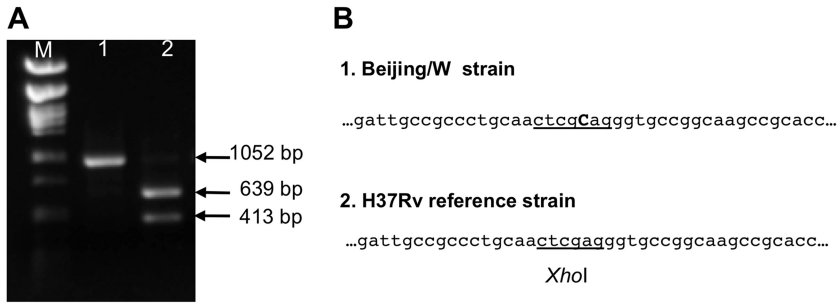

FIG 2 Screening of the Tap^{580} insertion in Beijing strains. (A) DNA gel electrophoresis showing PCR product with primers ctap9 and ctap10 digested with XhoI. M, size marker, lambda DNA digested with PstI; 1, *M. tuberculosis* GC1237 (Beijing); 2, *M. tuberculosis* H37Rv (non-Beijing). (B) Sequence of the region of the *Rv1258c* gene in a Beijing strain (panel 1) and *M. tuberculosis* H37Rv strain (panel 2) (GenBank accession no. AL123456.2).

non-Beijing strains, hence validating the method for screening the Tap^{580} insertion. In addition, a deletion of 4 nucleotides spanning positions 835 and 838 of the *Rv1258c* gene was found in one of the Beijing samples, which is located downstream of the in-frame stop codon in positions 694 to 696 produced by the Tap^{580} insertion.

Detection of the *Rv1258c* nucleotide insertion in clinical samples. In order to assess the usefulness of this technique for detecting Beijing strains directly in clinical samples, a blind panel of 30 respiratory specimens was assembled by the participating hospitals. This panel included 29 respiratory samples with diverse bacillary loads and that were culture-positive for *M. tuberculosis*, and one sample containing the nontuberculous mycobacterial species *M. fortuitum*. After isolating DNA from the sputum samples, the 1,052-bp PCR product was successfully amplified from 28 samples and digested with XhoI endonuclease. Out of these 28 samples, 26 were identified as containing non-Beijing *M. tuberculosis* strains, and two samples were identified as containing *M. tuberculosis* strains of the Beijing family, one of which was detected in a specimen with low bacillary load. The genetic lineage identification of the strains included in the panel was confirmed by the two hospitals that had designed the blind panel. The 1,052-bp PCR product could not be amplified from two sputum samples; one contained *M. fortuitum*, hence demonstrating the specificity of this method for detecting *M. tuberculosis*, and the second sample had a low bacillary load. Since eight of the nine samples with low bacillary load could be processed by this protocol, we assume that the failure to obtain the PCR amplification product with this sample might be due to other reasons.

The precise effect of this nucleotide insertion in the physiology of *M. tuberculosis* Beijing isolates remains to be fully elucidated. Recently, the role of the Rv1258c efflux pump in drug tolerance in *M. tuberculosis* has been reported (28). Since the identified single-nucleotide insertion in the *Rv1258c* gene in Beijing isolates results in a truncated, and most probably inactive, protein, this suggests that isolates carrying this nucleotide insertion might have a disadvantage in terms of drug resistance and drug tolerance compared with others carrying a fully functional Rv1258c transporter. However, since Beijing isolates are frequently associated with a higher propensity to acquire drug resistance, it is conceivable that other mutations actually compensate for the loss of Rv1258c in this family of *M. tuberculosis* isolates. Examples of compensatory mutations occurring following the acquisition of drug resistance-associated mutations have been described for *M. tuberculosis* (29).

In summary, a new genetic polymorphism of *M. tuberculosis* Beijing strains has been used to develop a simple and reliable technique for identifying isolates of this family. Given the higher transmissibility rate of this family of strains, which in addition are more prone to developing drug resistance, methods for rapid identification constitute a very important tool for the control of outbreaks caused by isolates of the Beijing family. This technique is fast, since it can be performed directly on clinical specimens and there is no need to culture strains; this makes it an ideal method for being implemented in those settings where routine culture of clinical samples cannot be done. In addition, this technique is easy to carry out and does not require sophisticated equipment, as only a thermocycler and DNA electrophoresis system are needed. In addition, samples can be processed individually or in groups of any size, and this can easily accommodate the workflow of a clinical laboratory without the need to process samples in groups of a fixed number. All this, along with the specificity for both the detection of *M. tuberculosis* and the identification of the Beijing family of strains, makes it ideal for implementation in clinical laboratories, especially in those settings with a high incidence of tuberculosis infections caused by this family of strains.

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