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Molecular Characterization of *Mycobacterium tuberculosis* H37Rv/Ra Variants: Distinguishing the Mycobacterial Laboratory Strain†

P. BIFANI,[1,2] S. MOGHAZEH,[1] B. SHOPSIN,[1,2] J. DRISCOLL,[3]
A. RAVIKOVITCH,[1] AND B. N. KREISWIRTH[1]*

Public Health Research Institute Tuberculosis Center[1] and Department of Microbiology, New York University School of Medicine,[2] New York, New York 10016, and Wadsworth Center, New York State Department of Health, Albany, New York 12201-2002[3]

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The *Mycobacterium tuberculosis* strains H37Rv and H37Ra are the most commonly used controls for *M. tuberculosis* identification in the clinical and research laboratory setting. To reduce the likelihood of misidentification and possible cross-contamination with this laboratory neotype, it is important to be able to distinguish H37 from clinical isolates. To provide a reference for identifying H37, we used multiple molecular techniques to characterize H37 strains, including 18 of the most frequently used variants available through the American Type Culture Collection. Isolates were genotyped using gene probes to IS*6110* and IS*1085*. In addition, we performed polymorphic GC-rich sequence typing (PGRS), spoligotyping, determination of variable number of tandem repeats (VNTR), and PCR amplification of the *mtp40*, *msx4*, and *mpp8* polymorphic regions. Southern hybridization with IS*6110* provided the most discrimination, differentiating the 18 H37 isolates into 10 discrete patterns made up of 9 H37Rv variants and 1 H37Ra variant. PGRS, IS*1085*, *mpp8*, and spoligotyping were not able to distinguish any H37 variants, while VNTR and *msx4* discriminated two. Only IS*6110* and spoligotyping could distinguish the H37 strain from clinical isolates. In summary, spoligotyping and IS*6110* provide a rapid and accurate way to identify H37 contamination, though IS*6110* can, in addition, classify many of the H37 variants that would otherwise require phenotypic segregation.

The genotyping of *Mycobacterium tuberculosis*, primarily for outbreak identification, has become a model for the application of strain typing in the field of molecular epidemiology. In the clinical mycobacteriology laboratory, strain typing has been essential in the identification of laboratory cross-contamination (1, 2, 5, 16, 25), an almost impossible task prior to the inception of molecular techniques. The source of laboratory cross-contamination can be a clinical sample (3, 15, 16) or often the *M. tuberculosis* control strain maintained by the clinical mycobacteriology laboratory (12, 13). In this regard, the virulent and attenuated H37 variants are the most commonly used control isolates and thus are a major source of false-positive results in *M. tuberculosis* identification as well as cross-contamination (13). The purpose of this study is to provide the mycobacteriologist with a molecular guide for discriminating H37 from clinical isolates in the genotyping laboratory.

The strain H37 was originally isolated in 1905 and gained attention for its noted virulence in the guinea pig model, a distinctive characteristic used in the classification of "human tuberculosis" in the early 1900s. In 1934, H37 was dissociated into "virulent" (Rv) and "avirulent" (Ra) strains (18, 24). The original 1905 H37 isolate was then discontinued, and the H37Rv and H37Ra isolates have been maintained at the Trudeau Institute ever since. Several drug-resistant derivatives have been generated during the years, accounting in part for the 18 H37 variants available through the Trudeau Mycobacterial Collection (TMC) and the American Type Culture Collection (ATCC). Hence, there are 15 H37Rv and 3 H37Ra progenies maintained at the Trudeau Institute and the ATCC.

Although H37 variants are widely used as reference strains in mycobacteriology and molecular biology laboratories, their IS*6110* patterns are often mistaken for clinical isolates displaying similar fingerprint patterns (unpublished data). In this respect, it is essential for both patient care and tuberculosis control to be able to properly recognize and genotype all possible H37 variants. To do so, we have employed several of the most common *M. tuberculosis* typing techniques in order to characterize the 18 H37 variants available through the ATCC. The results indicate that both spoligotyping and IS*6110* provide a rapid means of distinguishing H37 strains from clinical isolates. In addition, IS*6110* DNA fingerprinting analysis further discriminates the collection into 10 distinct H37 variants.

MATERIALS AND METHODS

***M. tuberculosis* reference strains.** The 18 different catalogued H37 variants were purchased from the ATCC. The strains were deposited at the ATCC as follows: 25177 (C. L. Larson, University of Montana, lot 1-23-69), 35618 (A. G. Karlson, Mayo Clinic, lot 1-21-70), and 27294 (G. P. Kubica, Trudeau Laboratories, lot 1-27-72). All other strains (35820, 35821, 35822, 35823, 35824, 35825, 35826, 35827, 35828, 35829, 35830, 35835, 35836, 35837 and 35838) were deposited by the Trudeau Institute in lot 2-01-85 (data were kindly provided by the ATCC). In addition, the primary collection of H37 variants was also received, as a kind gift of R. North, from the TMC, Trudeau Institute, Saranac Lake, N.Y. TMC and ATCC reference numbers and susceptibility data are shown in Table 1.

***M. tuberculosis* clinical isolates.** A search of the IS*6110* fingerprint database maintained at the Public Health Research Institute Tuberculosis Center (TB Center) (n = 11,000) identified 131 H37Rv and H37Ra isolates which matched at least one of the nine H37Rv and one H37Ra patterns reported in this study. The fingerprint search was conducted using each of the 10 possible H37Rv and H37Ra patterns as a prototype. The TB Center database includes approximately 8,600 isolates from New York City and New Jersey, while the remaining samples are from seven additional states in the United States and from the former USSR, Singapore, South Africa, Romania, Egypt, Israel, Venezuela, Honduras, Mexico, India, Chile, the Czech Republic, and Kenya.

Genotyping by IS*6110*. Chromosomal DNA extraction and strain typing by IS*6110* was performed according to the standard method using the right-side

* Corresponding author. Mailing address: Public Health Research Institute Tuberculosis Center, 455 First Ave., New York, NY 10016. Phone: (212) 578-0850. Fax: (212) 578-0853. E-mail: barry@phri.nyu.edu.

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TABLE 1. H37Rv and H37Ra collection[a]

ATCC no.	TMC no.	Genotype	Phenotype	Strain origin	Source or reference(s) (yr)
Rv					
25618	H37Rv	Rv_9	Pan^s	A. G. Karlson, Mayo Clinic	ATCC (1992)
27294	102(Rv)	Rv_7	Pan^s	Dissociated in 1934	18, 24
35820	301(Rv)	Rv_1	Str^r	Mutant of TMC102	21
35821	302(Rv)	Rv_2	Pas^r	Mutant of TMC102	W. Steenken (1948)
35822	303(Rv)	Rv_8	Inh^r	Mutant of TMC102	29
35823	304(Rv)	Rv_3	$Str^r Inh^r$	Mutant of TMC102	TMC (1954)
35824	305(Rv)	Rv_5	$Pas^r Str^r$	Mutant of TMC102	20
35825	306(Rv)	Rv_6	$Pas^r Str^r Inh^r$	Mutant of TMC102	TMC (1961)
35826	307(Rv)	Rv_8	Cyc^r	Mutant of TMC102	19
35827	309(Rv)	Rv_8	Kan^r	Mutant of TMC102	22
35828	311(Rv)	Rv_8	Pza^r	Mutant of TMC102	29
35829	313(Rv)	Rv_4	Tac^r	Mutant of TMC102	29
35830	314(Rv)	Rv_8	Eth^r	Mutant of TMC102	17
35837	330(Rv)	Rv_7	Emb^r	Mutant of TMC102	TMC (1970)
35838	331(Rv)	Rv_7	Rif^r	Mutant of TMC102	TMC (1970)
Ra					
35835	326(Ra)	Ra_1	Inh^r	Mutant of TMC201	TMC (1961)
35836	327(Ra)	Ra_1	Str^r	Mutant of TMC201	TMC (1949)
25177	201(Ra)	Ra_1	Pan^s	Dissociated in 1934	18, 24

[a] H37 variant list. Abbreviations: Cyc, cycloserine; Emb, ethambutol; Eth, ethionamide; Inh, isoniazid; Kan, kanamycin; Pas, *para*-aminosalicylic acid; PZA, pyrazinamide; Rif, rifampin; Str, streptomycin; Tac, thioacetazone (17–24, 29). Pan^s, pan-susceptible.

hybridization probe (IS*6110*-3′-probe) (27). The same membrane was rehybridized with the left-side IS*6110* probe (IS*6110*-5′-probe), the direct repeat (DR) probe, and the insertion sequence IS*1085*.

Genotyping by using PGRS probe. Chromosomal DNA was restricted with *Alu*I and hybridized with the polymorphic GC-rich repetitive sequence (PGRS) probe (GenBank accession no. M95490) (14). All other electrophoretic and hybridization conditions were the same as for IS*6110* genotyping (26).

Spoligotyping. The DR of the extracted *M. tuberculosis* DNA was amplified by PCR and analyzed according to the spoligotyping protocol as described by Kamerbeek and colleagues (8). The *Alu*I-digested DNA membranes generated for PGRS typing were also probed with the DR probe to confirm spoligotyping results (27).

Determination of VNTR. The variable number of tandem repeat (VNTR) loci ETR-A to ETR-E were determined as described by Frothingham and Meeker-O'Connell (7). Briefly, the five selected loci were amplified by PCR and analyzed on a 2% agarose gel.

PCR amplification of the polymorphic fragments *msx4*, *mpp8*, and *mtp40*. Amplicons to segments *msx4*, *mpp8*, and *mtp40* were generated and compared from all 18 H37 variants. The polymorphic segments *msx4* and *mpp8* containing two DR sequences were PCR amplified with primer pairs SX1-SX2 and PP3-PP4, respectively, as described by Namwat et al. (11). PCR amplification of the *mtp40* fragment was accomplished using primers PT1 and PT2 (6).

Computer analysis of fingerprint patterns. The IS*6110* hybridization patterns were electronically digitized and compared with a pattern-matching computer program on a Sun Sparc5 workstation using a Bioimage Whole Band Analyzer (software version 3.4; Genomic Solutions, Ann Arbor, Mich.). The Jaccard matching method and unweighted-pair-group method using arithmetic averages (UPGMA)-average linkage clustering was used to identify related patterns, in accord with the protocol of the Centers for Disease Control and Prevention, The National Tuberculosis Genotyping and Surveillance Network.

RESULTS

Eighteen H37 variants available through the ATCC and their respective parent strains from the Trudeau Institute were typed by the now standard IS*6110* Southern blot hybridization analysis (26). A total of 10 distinct fingerprint patterns were identified (Fig. 1). The nine patterns associated with the H37Rv strains were assigned the genotypes Rv_1 through Rv_9 and all three H37Ra strains (35835, 35836, and 25177) shared the same Ra_1 fingerprint pattern (Table 1). The Rv variants were Rv_1 (strain 35820), Rv_2 (strain 35821), Rv_3 (strain 35823), Rv_4 (strain 35829), Rv_5 (strain 35824), Rv_6 (strain 35825), Rv_7 (strains 35837, 35838, and 27294), Rv_8 (strains 35822, 35826, 35827, 35828, and 35830), and Rv_9 (strain 35618). The number of IS*6110* hybridizing bands ranged from 14 (Rv_4) to 16 (Rv_7) bands for H37Rv and was 16 bands for H37Ra (Ra_1; Fig. 1).

The spoligopattern of H37 variants was unique (spoligotype S00001; Fig. 2). The S00001 spoligopattern has only been observed in isolates of H37 variants and clinical samples determined to have been cross-contaminated by H37 strains. VNTR patterns were determined by analysis of the products of the PCR amplification of the five chromosomal loci ETR-A to ETR-E. All 18 ATCC isolates displayed the same VNTR pattern (33433), except for Rv_5. PGRS-typing, spoligotyping, and

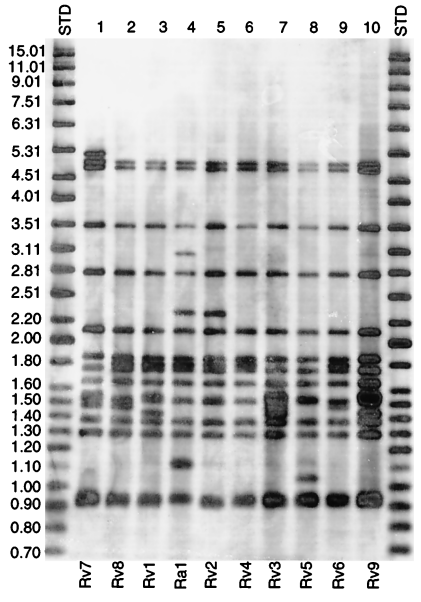

FIG. 1. IS*6110* hybridization patterns of H37. Ten different IS*6110* patterns were identified from the 18 H37 variants available through the ATCC and the TMC.

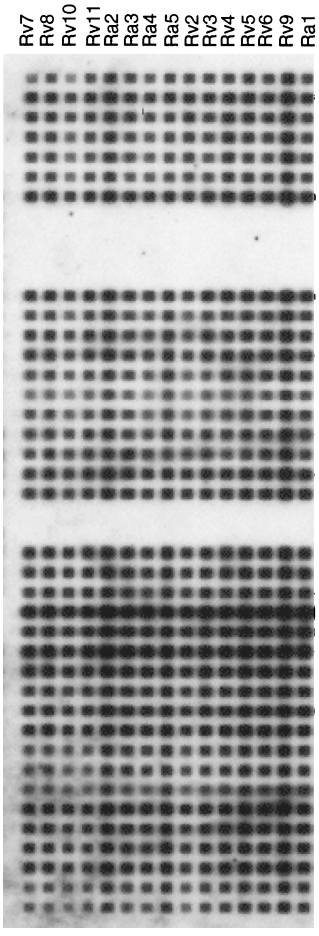

FIG. 2. Spoligotype pattern of H37 variants. All 18 H37 ATCC and TMC samples, as well as 5 H37 laboratory variants, were found to have the same pattern. This pattern was not seen in a spoligotype database of 2,400 samples other than for H37 control samples.

VNTR analysis did not differentiate the 18 H37 variants within the TMC and ATCC isolates (see Fig. 4).

PCR amplification of three other polymorphic regions, the two repetitive sequences *msx4*, *mpp8*, and *mtp40*, and hybridization with IS*1085*, could not be used to distinguish H37 variants from other genetically unrelated *M. tuberculosis* strains. The *msx4* PCR products of all H37 variants were shown to be 437 bp in length, with the exception of strain ATCC 35825, which generated a 720-bp amplicon (data not shown).

Genotyping clinical isolates. Searching the IS*6110* DNA fingerprint pattern database at the TB Center (n = ~11,000) using Rv_1 to Rv_9 and Ra_1 as prototypes, identified 131 IS*6110* patterns that matched exactly at least 1 of the 18 H37 variants. A total of 102 samples matched H37Ra type Ra_1, 27 matched H37Rv type Rv_7, and 1 each matched Rv_1 and Rv_8. Of these, 45 samples identified as H37Ra were previously reported as known cases of cross-contamination following an investigation by the New York City Department of Health (13). Three H37Ra samples were from outside the United States. The New York State Department of Health confirmed an additional 14 H37Ra strains to be cases of laboratory cross-contamination (12). Of the remaining 69 cases, 37 were confirmed to be contamination by the source laboratories, and the remaining are being investigated by the responsible entities.

Five additional samples had a similar but not exact match with one of the H37Rv or H37Ra variants. The five samples differed from Rv_1 to Rv_9 and Ra_1 by one or two hybridizing bands according to IS*6110* analysis (Fig. 3). These samples were designated Rv_{10} and Rv_{11} and Ra_2 through Ra_4 (Fig. 3). Further analysis of all five isolates by the IS*1085*, VNTR, PGRS, and spoligotype methods failed to distinguish these samples from the reference TMC-ATCC prototypes (Fig. 4). The PGRS profile of H37 Rv_1, Rv_7, Rv_8, Rv_{10} and Rv_{11}, and Ra_1 and Ra_4 can be seen in Fig. 4.

Two of the strains, designated Rv_{10} and Ra_2 (Fig. 3), were received as part of a large cluster of H37Rv and H37Ra cross-contamination investigations (13). Furthermore, strains Rv_{10} and Ra_2 were very closely related to the standard laboratory reference strains Rv_1 and Ra_1, respectively. Rv_{10} and Ra_2 differ from Rv_1 and Ra_1 by the deletion of one IS*6110* insertion (Fig. 3). Another variant (Rv_{11}) was routinely used as a reference strain by a clinical laboratory and was supplied for fingerprint analysis as part of a control experiment. Cross-contamination by the remaining two H37 isolates (Ra_3 and Ra_4) was confirmed by reviewing laboratory and clinical data pertaining to these two cases. Thus, it can be inferred that Rv_{10}, Rv_{11}, Ra_2, Ra_3, and Ra_4 are variants of an H37 reference strain which have further evolved in the lab.

DISCUSSION

In the speciation and susceptibility testing of *M. tuberculosis* in the clinical laboratory, suspicion of contamination is heightened by the finding of an unusually high number of positive

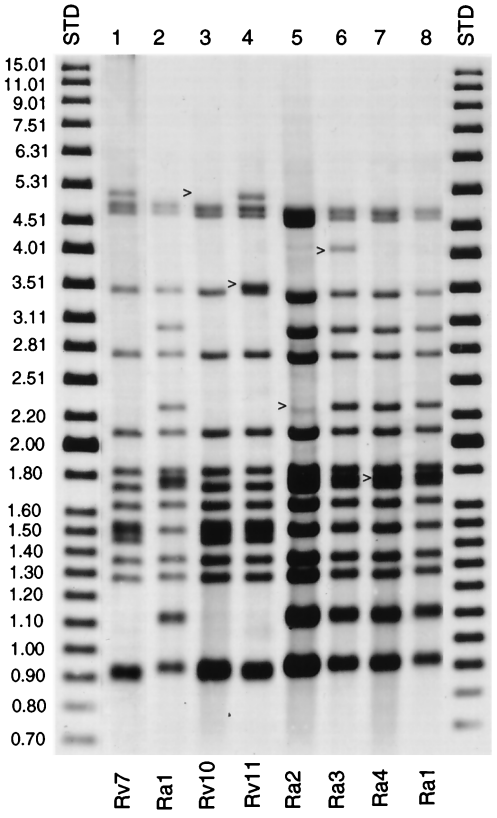

FIG. 3. IS*6110* hybridization patterns of Rv_7, Ra_1, and five H37 polymorphic variants. As indicated, Rv_{10} and Rv_{11} (lanes 4 and 5) differ from Rv_7 (lane 2) by the loss and addition of an IS*6110* insertion, respectively (see arrows). Ra_2 (lane 6) has lost an IS*6110* insertion, while Ra_3 and Ra_4 (lanes 7 and 8) have gained an insertion compared to the reference strain Ra_1 (lane 3 and 9).

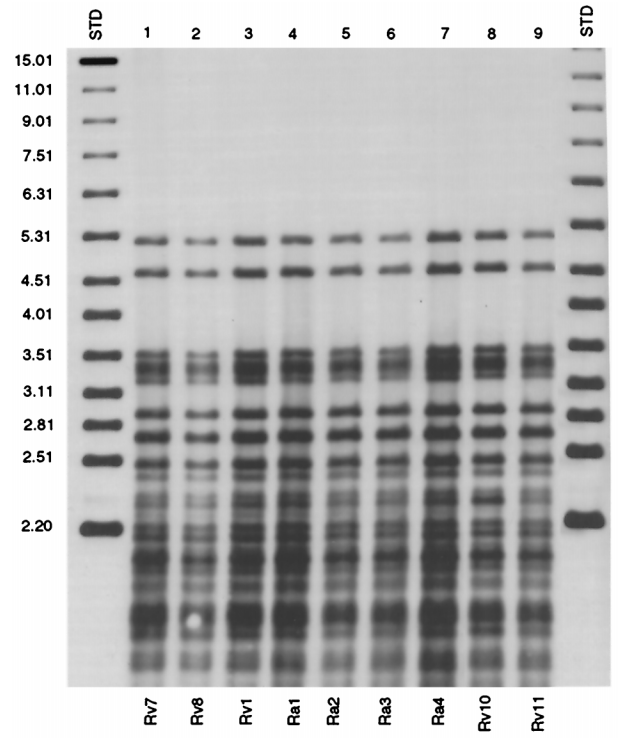

FIG. 4. Southern hybridization of *Alu*II-digested chromosomal DNA with the PGRS probe for strains Rv_7, Ra_1, and five H37 polymorphic variants. PGRS grouped all H37 variants but could not differentiate between them.

cultures per time period and by inconsistencies between the patient's clinical presentation and his or her laboratory results (10). Suggested cases of contamination are forwarded to a limited number of genotyping laboratories to perform molecular analysis to confirm or reject the possibility of contamination, primarily on a case-by-case basis (1, 2, 5, 16, 25). Often, genotyping and mycobacteriology laboratories are unaware of which H37 variant they are employing as controls, confounding the identification of contaminants. In this study, we have provided a collection of patterns for H37 and its variants for use as a reference by genotyping laboratories.

Genotyping analysis of H37 variants. All 18 ATCC H37 variants had the same spoligotype pattern, designated S00001. Although spoligotype pattern S00001 did not discriminate one ATCC H37 variant from another, it proved definitive in distinguishing the ATCC H37 collection from all other clinical isolates analyzed by this technique ($n \approx 2{,}400$).

Based on the similarity of the IS*6110* DNA fingerprint patterns, all H37 variants were grouped as related. IS*6110* discriminated the H37 variants, identifying 9 distinct yet similar patterns (Rv_1 to Rv_9) of 15 possible phenotypically (drug resistance profile) diverse H37Rv isolates and a single pattern for all 3 H37Ra (Ra_1) isolates. The differences in IS*6110* fingerprint patterns between H37Rv and H37Ra have been previously examined by restriction analysis using four different endonucleases (9), and the molecular bases for the alternate IS*6110* patterning have been investigated by comparative sequence analysis using the bacterial-artificial-chromosome method (4). Unfortunately, since the vast majority of studies involve H37 isolates, the above-mentioned investigations failed to properly identify the H37Rv and H37Ra used (4, 9).

The H37 IS*6110* patterns shown in this study may be used as a reference for genotyping analysis; however, it is conceivable that cultures that have been maintained over the years in different laboratories or else different culture lots available from the ATCC might have evolved additional related IS*6110* patterns.

The accuracy of spoligotyping and IS*6110* fingerprint analysis in the identification of H37 variants was evaluated by comparison with other well-established molecular techniques. As with spoligotyping, PGRS and VNTR analyses were found to be nondiscriminating within the 18 ATCC H37 variants. Thus, the spoligotyping, PGRS, and VNTR methods can group the ATCC H37 collection as shown but cannot distinguish any variants. In addition, when H37 PGRS and VNTR patterns were compared to our database (PGRS, $n > 600$; VNTR, $n > 400$), a close similarity was noted with a large cluster of clinical isolates (unpublished data), rendering detection of the H37 variants by one of these two techniques unreliable. In agreement with VNTR analysis of an unspecified H37Rv and H37Ra in another work (7), we found that all 18 TMC-ATCC H37 variants (except for Rv_5) share the same VNTR pattern for loci ETR-A to ETR-E, i.e., strain 33433. Polymorphisms encountered in amplicons, *msx4*, *mpp8*, and *mtp40* could not be used to discriminate the H37 variants from other clinical isolates, in agreement with other studies (11, 28).

Identifying H37 cross-contamination among clinical isolates. The ability to discriminate between the H37 variants and real clinical samples has important public health implications. In this study, the IS*6110* fingerprint patterns of 131 clinical isolates, most of which were confirmed contaminants, matched that of one of the reference H37 strains. Random spoligotyping, PGRS, and VNTR analyses confirmed the relatedness of these 131 isolates to the reference ATCC H37 variants.

In addition, two H37Rv and three H37Ra IS*6110* patterns (Rv_{10} and Rv_{11} and Ra_2 to Ra_4), which are distinct from but related to the ATCC collection, were identified among clinical specimens from our database of 11,000 fingerprints. These five isolates, also confirmed cases of laboratory contamination, were found to have the same spoligotype, PGRS, and VNTR as the TMC-ATCC isolates, and we infer that the isolates have evolved in the clinical laboratory from one of the reference strains.

However, investigators should be aware that, given the origins of H37, it is possible that "true" clinical isolates exist with an identical spoligotype and similar IS*6110* pattern as the reference strain H37. Thus, while genotyping may be used to initiate or direct investigation, clinical decisions regarding contamination should be based on a combination of molecular and medical information.

Taken together, analysis of our clinical *M. tuberculosis* collection as well as ATCC isolates indicates that a combination of spoligotyping and IS*6110* fingerprinting has proven to be a reliable tool in the proper identification of H37 cross-contamination. Unlike PGRS analysis, the H37 spoligopatterns (S00001) were unambiguous, making interpretation consistent. IS*6110* fingerprinting should be used to confirm the proper identification of an H37 isolate.

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