# Thr202Ala in thyA Is a Marker for the Latin American Mediterranean Lineage of the Mycobacterium tuberculosis Complex Rather than Para-Aminosalicylic Acid Resistance

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# Thr202Ala in *thyA* Is a Marker for the Latin American Mediterranean Lineage of the *Mycobacterium tuberculosis* Complex Rather than *Para*-Aminosalicylic Acid Resistance▽

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**Single nucleotide polymorphisms (SNPs) involved in the development of resistance represent powerful markers for the rapid detection of first- and second-line resistance in clinical *Mycobacterium tuberculosis* complex (MTBC) isolates. However, the association between particular mutations and phenotypic resistance is not always clear-cut, and phylogenetic SNPs have been misclassified as resistance markers in the past. In the present study, we investigated the utility of a specific polymorphism in *thyA* (Thr202Ala) as a marker for resistance to *para*-aminosalicyclic acid (PAS). Sixty-three PAS-susceptible MTBC strains comprising all major phylogenetic lineages, reference strain H37Rv, and 135 multidrug-resistant (MDR) strains from Germany (comprising 8 PAS-resistant isolates) were investigated for the presence of Thr202Ala. In both strain collections, the Thr202Ala SNP was found exclusively in strains of the Latin American Mediterranean (LAM) lineage irrespective of PAS resistance. Furthermore, PAS MICs (0.5 mg/liter) for selected LAM strains (all containing the SNP) and non-LAM strains (not containing the SNP), as well as the results of growth curve analyses performed in liquid 7H9 medium in the presence of increasing PAS concentrations (0 to 2.0 mg/liter), were identical. In conclusion, our data demonstrate that the Thr202Ala polymorphism in *thyA* is not a valid marker for PAS resistance but, instead, represents a phylogenetic marker for the LAM lineage of the *M. tuberculosis* complex. These findings challenge some of the previous understanding of PAS resistance and, as a consequence, warrant further in-depth investigations of the genetic variation in PAS-resistant clinical isolates and spontaneous mutants.**

*para*-Aminosalicylic acid (PAS) was one of the first drugs used in the treatment of tuberculosis (TB) but initially caused severe side effects, resulting in poor patient compliance. Thanks to a new formulation of the drug leading to improved gastrointestinal tolerance (5), PAS has since then become one of the components in second-line drug treatment to which even highly drug-resistant isolates are susceptible (3, 12, 16).

Rengarajan et al. (15) first reported that PAS targets the folate pathway: selection for PAS resistance using transposon mutagenesis led to the isolation of strains with transposon-disrupted *thyA* (*Rv2764c*) which exhibited a reduced thymidylate synthase activity. Unlike the wild-type *thyA* from *Mycobacterium bovis* BCG (MtbBCG), the expression of the mutated *thyA* gene failed to rescue a *thyA*-negative phenotype in *Escherichia coli*. Similarly, only the expression of the wild-type MtbBCG *thyA* in the same *E. coli* background restored sensitivity to trimethoprim (TMP) (15).

Complementing these results, two mutations in *thyA* (Thr202Ala in strains R1 and R3 and Arg222Gly in strain R2) were identified in clinical PAS-resistant *Mycobacterium tuberculosis* strains. Both alleles failed to fully restore sensitivity to TMP in the *thy*-negative *E. coli* background described above. Lastly, the effect of expressing the wild-type *thyA* gene as well as both clinical alleles in one of their original transposon-disrupted *thyA* MtbBCG mutants was investigated. Again, only the expression of the wild-type gene completely restored susceptibility to PAS (15).

The involvement of *thyA* in PAS resistance has also been supported by a study of clinical strains from China. Of the 51 PAS-sensitive and 44 PAS-resistant strains investigated, only the resistant strains showed mutations in *thyA*. However, these accounted for only 36% of the phenotypic resistance, pointing to one or several unknown resistance mechanisms (20).

In the attempt to identify further the mechanism leading to PAS resistance, six genes of the folate and thymidine biosynthetic pathways have been analyzed by Mathys et al. (11). Although some mutations were identified in these genes, they did not appear to be relevant for PAS resistance. Instead, the detection of 25 distinct mutations in *thyA* reaffirmed the role of this gene. Notably, the Thr202Ala mutation discussed earlier was the mutation most frequently identified in 23 clinical isolates and in 1 spontaneous mutant. It was predicted to have a structurally destabilizing effect (11).

Our initial analysis of all previously sequenced *M. tuberculosis* complex (MTBC) strains revealed that this single nucleotide polymorphism (SNP) was present in the following

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strains: 98-R604, F11, GM 1503, KZN 506, KZN 605, KZN 1435, KZN 2475, and KZN 4207. Given that these eight genomes encompass different subfamilies of the Latin American Mediterranean (LAM) genotype, on the basis of their spoligotype patterns, which were found to accurately define this group (1), this indicated that *thyA* Thr202Ala might be a specific mutation or even a marker for this lineage.

To investigate this striking finding and to define the actual role of *thyA* Thr202Ala in PAS resistance development, we carried out a more systematic investigation. Sequence analyses of a fragment of the *thyA* gene of MTBC strains of two different collections, one comprising 63 PAS-susceptible MTBC strains of all major phylogenetic lineages and reference strain H37Rv and the other comprising 135 multidrug-resistant (MDR) strains from Germany, 8 of which were PAS resistant, was carried out. In addition, PAS MIC determinations and growth curve analyses in 7H9 medium with increasing PAS concentrations were performed for selected strains with and without the mutation.

## MATERIALS AND METHODS

**Mycobacterial strains and growth conditions.** A total of 198 MTBC strains were included in this study. One set of strains contained 63 isolates from a reference collection comprising major phylogenetic lineages, such as Beijing, Haarlem, Delhi/central Asian (CAS), and East African Indian, and 3 LAM strains, as well as the reference strain *M. tuberculosis* H37Rv ATCC 27294 (Fig. 1). All these strains were susceptible to PAS. The second set of strains analyzed contained 135 MDR strains that were collected in Germany during the years 2006 and 2007. Out of these strains, 127 were PAS susceptible (10 belonged to the LAM genotype, 117 to other genotypes) and 8 isolates were PAS resistant (one belonged to the LAM genotype, 7 to other genotypes). All strains were included in the sequencing analyses of a part of the *thyA* gene. A subset of selected strains was used for the determination of the MIC as well as for performing growth curve analyses in the presence of various PAS concentrations. Primary isolation and cultivation were done at the Supranational Reference Laboratory in Borstel, Germany, as described previously (9).

**Drug susceptibility testing.** PAS drug susceptibility testing was performed at the Supranational Reference Laboratory in Borstel by using the proportion method on Löwenstein-Jensen (LJ) medium with 0.5 and 1.0 mg/liter PAS (4). The determination of PAS MICs was also carried out by using LJ medium. The concentrations tested were 0.063, 0.13, 0.25, 0.5, and 1.0 mg/liter PAS. Growth curve analyses were performed in 7 ml 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (10%) and Tween 80 (0.05%) in the presence of 0, 0.13, 0.25, 0.5, 1.0, and 2.0 mg/liter PAS at 37°C in a rolling device (PAS was added at an optical density at 600 nm [$OD_{600}$] of 0.1). Mycobacterial growth was monitored spectrophotometrically (OD at 600 nm) every day for 9 consecutive days in triplicate.

**Molecular strain typing.** Extraction of genomic DNA from mycobacterial strains and DNA fingerprinting using IS*6110* as a probe were performed according to a standardized protocol, as described elsewhere (18). Additionally, all isolates were analyzed by the spoligotyping technique, as described by Kamerbeek et al. (8). Twenty-four-locus mycobacterial interspersed repetitive-unit–variable-number tandem-repeat (MIRU-VNTR) typing was done as published previously (13, 17). The molecular typing data were analyzed by using Bionumerics software (version 6.1; Applied Maths), as instructed by the manufacturer. Phylogenetic classification of the strains was carried out by using the MIRU-VNTRplus webpage (1, 19).

**PCR and sequencing.** DNA amplification of a 331-bp fragment of *thyA* was performed using primers thyA_5′ (position +432; 5′-GCCCTGTCATGCGTTC TT-3′) and thyA_3′ (position +762; 5′-ATGCGGATCGTAGTTCTTCAC-3′). The conditions applied were (i) initial denaturation at 95°C for 5 min, (ii) denaturation at 94°C for 30 s, (iii) annealing at 55°C for 30 s, (iv) elongation for 20 s at 72°C, and (v) terminal elongation for 5 min at 72°C. Steps ii to iv were repeated 35 times. The PCR products thus obtained were sequenced using an ABI 3130xl genetic analyzer (Applied Biosystems, CA) and an ABI BigDye Terminator cycle sequencing kit (version 3.1), according to the manufacturer's instructions. Analysis of sequence data was performed using the DNAStar Lasergene (version 8.0) program with *M. tuberculosis* H37Rv (GenBank accession number AL123456.2) as the reference sequence.

**Genome analysis.** The nucmer and show-snps functions of the MUMmer (version 3.20) program were used to determine SNPs in strain F11 (GenBank accession number CP000717.1) and in strains KZN 2475 and KZN 506 (7, 10). In addition, SNPs from the genomes of the following strains were called using BLAST analysis (2): 98-R604 INH-RIF-EM (GenBank accession number ABVM00000000.1), GM 1503 (GenBank accession number ABQG00000000.1), KZN 605 (GenBank accession number ABGN00000000.1), KZN 1435 (GenBank accession number CP001658.1), and KZN 4207 (GenBank accession number ABGL00000000.1).

## RESULTS

Following our initial genome analysis, the aim of the present study was to investigate if the Thr202Ala SNP in *thyA* is a marker for resistance to PAS, as suggested previously (11, 15), or, rather, represents a phylogenetic marker for the LAM lineage. To address this question, first, we tested different strain collections for the presence of the SNP and, second, performed MIC and growth curve analyses of selected strains with and without the *thyA* Thr202Ala SNP.

**Sequence analyses of *thyA* position 202.** PCR amplification and DNA sequence analysis of the 331-bp fragment of *thyA* comprising Thr202Ala was successful for all strains included in the study. All sequences obtained were compared with the H37Rv reference sequence to identify the presence of the polymorphism. Among the 63 PAS-susceptible *M. tuberculosis* complex strains comprising the major phylogenetic lineages of the MTBC (ranging from the *M. tuberculosis* LAM, Haarlem, East African Indian, Delhi/CAS, and Beijing lineages to the *M. africanum* West African 1 and West African 2 lineages), only the 3 strains belonging to the LAM lineage had the Thr202Ala SNP (Fig. 1), further supporting the hypothesis that this SNP is LAM specific.

To further corroborate these findings, we analyzed a total of 135 MDR strains collected in Germany during the years 2006 and 2007. Out of those strains, 8 isolates were PAS resistant (1 belonged to the LAM lineage, 7 to other phylogenetic lineages) and 127 strains were PAS susceptible (10 belonged to the LAM lineage, 117 to other lineages). Confirming the findings from the analysis of the PAS-susceptible reference collection, all 11 LAM strains carried the SNP at *thyA* position 202, irrespective of PAS resistance, whereas all other strains belonging to various different genotypes showed the wild-type sequence at this position (data not shown). This also applied to the 7 PAS-resistant strains that did not belong to the LAM lineage.

**MIC determination and growth curves.** Although our data demonstrate that the SNP at *thyA* position 202 constitutes a phylogenetic marker for the LAM genotype, it might still be possible that it confers to LAM strains a slightly enhanced intrinsic PAS resistance level compared to that for non-LAM strains that is not detected by routine drug susceptibility testing. To analyze if *thyA* Thr202Ala actually leads to low-level resistance to PAS, MICs for a selection of LAM and non-LAM strains were determined on LJ medium (PAS concentrations, 0.063, 0.13, 0.25, 0.5, and 1.0 mg/liter) by conventional susceptibility testing (Table 1). The experiment included two strains belonging to the LAM genotype (7968/03 and 8885/05), as well as one Beijing strain (1934/03), one Haarlem strain (9532/03), and the H37Rv reference strain (9679/00). For all strains analyzed, a PAS MIC of 0.5 mg/liter was determined, indicating

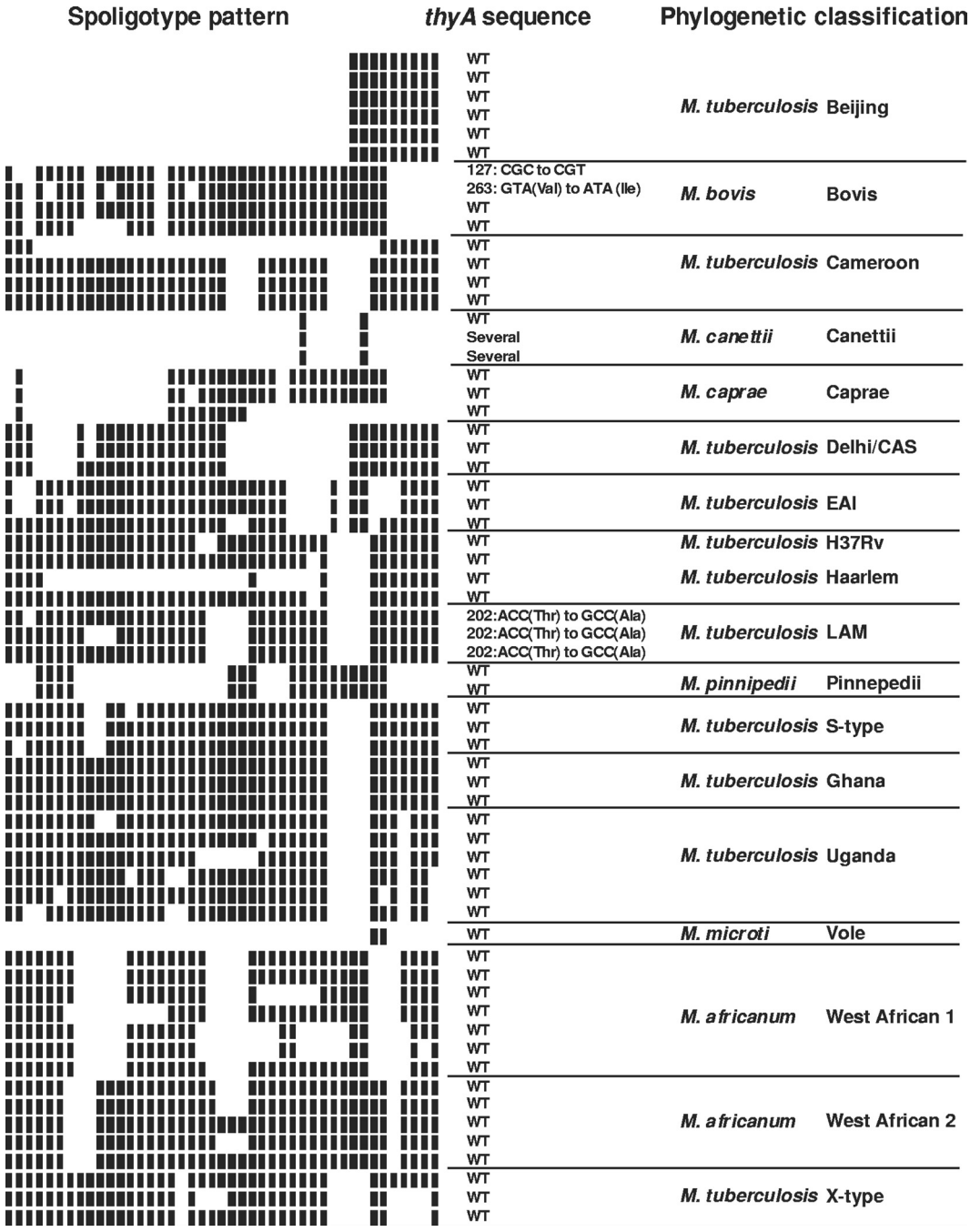


FIG. 1. Spoligotype patterns and presence of the *thyA* Thr202Ala polymorphism in 63 strains of a *Mycobacterium tuberculosis* complex reference collection, including *M. tuberculosis* reference strain H37Rv. The strains are ordered according to phylogenetic classification performed on the basis of the combined analysis of the 24-locus MIRU-VNTR typing results and the spoligotyping patterns, available at the MIRU-VNTRplus webpage (www.miru-vntrplus.org). Abbreviations: WT, wild type; EAI, East African Indian.

that the *thyA* SNP does not influence PAS susceptibility (Table 1). Additionally, growth curve analyses of the LAM and none-LAM strains described above were performed in 7H9 broth in the presence of increasing PAS concentrations to detect possible growth differences due to the presence of the *thyA* SNP. However, all strains tested were equally susceptible to all PAS concentrations tested and showed comparable growth curves (Fig. 2).

## DISCUSSION

The analyses performed in the present study clearly show that Thr202Ala (ACC → GCC) in *thyA* is not involved in the development of resistance to PAS in *M. tuberculosis* complex isolates but represents a phylogenetic marker for the LAM lineage. The first line of evidence was obtained from the fact that the SNP was present in all strains of the LAM lineage,

TABLE 1. Determination of MICs to PAS performed on Löwenstein-Jensen medium[a]

| Strain | Genotype | Amino acid at *thyA* position 202 | MIC (mg/liter) |
|---|---|---|---|
| 7968/03 | LAM | Ala (GCC) | 0.5 |
| 8885/05 | LAM | Ala (GCC) | 0.5 |
| 1934/03 | Beijing | Thr (ACC) | 0.5 |
| 9532/03 | Haarlem | Thr (ACC) | 0.5 |
| 9679/00 | H37Rv | Thr (ACC) | 0.5 |

[a] Selected strains were chosen from the reference collection representing the LAM genotype as well as two other genotypes (Beijing, Haarlem) and the reference strain *M. tuberculosis* H37Rv. Furthermore, the results of the sequencing analysis at *thyA* position 202 are shown.

irrespective of their resistance to PAS, and the fact that none of the PAS-resistant non-LAM strains carried the mutation. Furthermore, we could exclude a slightly enhanced level of PAS resistance in LAM strains by MIC and growth curve analyses. As such, *thyA* Thr202Ala does not render LAM strains naturally resistant to PAS.

These findings do not contradict the evidence presented by Zhang et al., as the mutations identified in China have not been described elsewhere (20). In contrast, our data can be only partially reconciled with the remaining two studies discussed in the introduction to the present work (11, 15).

Mathys et al. detected the *thyA* Thr202Ala polymorphism in resistant clinical *M. tuberculosis* isolates and in one spontaneous selection mutant and described it as a major PAS resistance mechanism (11). However, a closer look at the spoligotyping data presented in Table 1 of their paper (11) revealed that, in accordance with our findings, the majority of strains carrying the *thyA* Thr202Ala polymorphism belong to the LAM genotype (some strains could not be classified unambiguously on the basis of the spoligotype octal codes presented). Interestingly, 12 out of 15 strains of the P-strain family (all of which clearly belong to the LAM lineage and had the *thyA* Thr202Ala mutation) had an additional mutation in *thyA* (stop264Arg) which might, instead, be responsible for the development of PAS resistance in these strains (11).

Our results are more difficult to reconcile with the experimental analysis of mutants R1 and R3 (Thr202Ala) by Rengarajan et al. discussed in the introduction to the present work. One possibility might be that their findings were artificial due to the *E. coli* background or the expression vector that they used, and thus, those findings do not translate into phenotypic resistance in *M. tuberculosis* (15).

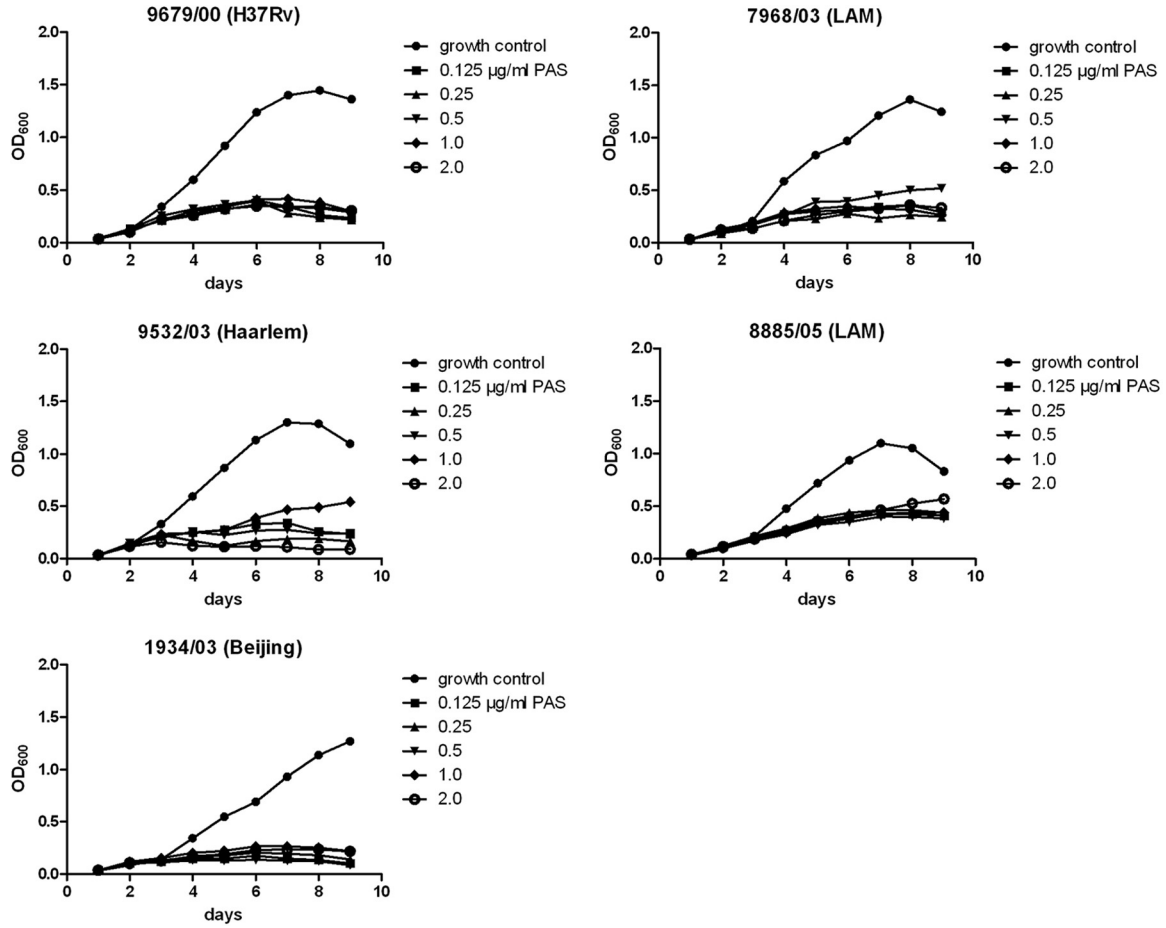


FIG. 2. Growth curves of five *M. tuberculosis* strains belonging to different genotypes in the presence of different PAS concentrations (0.125 to 2.0 mg/liter). Mean values of three independent experiments were plotted.

Despite these apparent contradictions, we demonstrated that *thyA* Thr202Ala should be regarded as a marker for the LAM genotype instead of PAS resistance. These results underline the importance of considering the MTBC population structure when new resistance mechanisms are defined by comparative sequencing. Indeed, we have already demonstrated in previous investigations that phylogenetic informative polymorphisms are present in both confirmed resistance genes (e.g., *embB*) and refuted candidate genes (*Rv2629*) (6, 14).

The fact that *thyA* Thr202Ala can no longer be considered a resistance marker has additional implications for our limited understanding of the genetic changes responsible for PAS resistance. For example, in the study of Mathys et al. (11), exclusion of *thyA* Thr202Ala further enhances the percentage of clinical isolates without any mutation in the six genes of the folate and thymidine biosynthetic pathways investigated. Consequently, unknown mechanisms play a more significant role in conferring resistance to PAS than was previously appreciated.

In conclusion, the results obtained in our study, despite contradicting previous findings, clearly highlight the relevance of *thyA* Thr202Ala as a newly identified marker for the LAM lineage and not for the development of PAS resistance. In light of these results, further efforts are needed to fully elucidate the molecular basis of PAS resistance.

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