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Relation of *Mycobacterium tuberculosis* mutations at *katG*315 and *inhA*-15 with drug resistance profile, genetic background, and clustering in Argentina

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ABSTRACT

We analyzed 362 isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* obtained countrywide for the presence of mutation at *katG*315 and *inhA*-15 in relation to genotype, pattern of phenotypic resistance to other drugs, and ability to spread. We found the following mutation frequencies: *katG*315MUT/*inhA-15*wt 53.0%, katG315wt/*inhA*-15MUT 27.4%, katG315wt/inhA-15wt 19.3%, and katG315MUT/inhA-15MUT only 0.3%. Mutation at *katG*315 associated with the LAM superfamily; mutation at *inhA*-15 associated with the S family and the T1 Tuscany genotype; the combination katG315wt/inhA-15wt associated with the T1 Ghana genotype. Isolates harboring *katG*315MUT/inhA-15wt tended to accumulate resistance to other drugs and were more frequently found in cluster; isolates harboring katG315wt/inhA-15wt were more frequently found as orphan isolates. Although epidemiological and host factors could also be modulating the events observed, in Argentina, the systematic genotyping of drug resistant clinical isolates could help to predict an enhanced risk of transmission and a propensity to develop resistance to increasing numbers of drugs.

1. Introduction

Much effort has been made globally to hamper the emergence of drug resistant tuberculosis (TB). Unfortunately, many drug resistant and multidrug resistant (MDR) *Mycobacterium tuberculosis* strains have managed to circumvent such efforts, proving able to infect, cause disease, acquire further drug resistance, and still spread around the world (Fenner et al., 2012). MDR-TB is caused by bacilli resistant at least to isoniazid (INH) and rifampicin (RIF). As these are the most effective frontline drugs against the disease, MDR-TB requires lengthened therapeutic schemes that include multiple drugs which are much more toxic and less effective. Extensively drug resistant (XDR) TB is an even more severe condition because it is MDR-TB with additional resistance to at least one fluoroquinolone and one second-line injectable drug, the most effective groups in this second line anti-TB drug armamentarium. Poly drug resistant TB is caused by bacilli displaying resistance to two or more drugs, except for the combined resistance to INH and RIF.

The so-called "canonical" mutations at *katG*315 and *inhA*-15 promoter region are the first and second most frequent mutations conferring INH resistance in *M. tuberculosis* globally, with regional variations. Different *M. tuberculosis* lineages also prevail in different regions of the world. Thus, the acquisition of drug resistance mutation might be driven by the genetic background of each strain and *M. tuberculosis* heterogeneity throughout the world might explain the observed regional variability in the frequencies of different resistance mutations (Fenner et al., 2012; Seifert et al., 2015). Previous work have postulated that mutation at position 315 of the *katG* gene confers high-level INH resistance and represents low or null fitness cost for *M. tuberculosis.* Indeed, in a given population, INH-resistant strains carrying mutation at this site were found to prevail over INH-resistant strains not carrying it (van Doorn et al., 2006). To a lesser degree, low fitness cost has been also attributed to mutation in the promoter region of the *inhA* gene at position -15, which confers low-level resistance to INH (Fenner et al., 2012; Gagneux et al., 2006).

In Argentina, two MDR *M. tuberculosis* strains have been transmitted epidemically for the last 20 years, each predominating in a distinct geographic setting. They are the M strain of the Haarlem family (H2 SIT2) and the Ra strain of the Latin American and Mediterranean family (LAM3 SIT33) (Aita et al., 1996; Ritacco et al., 1997). Together, they account for more than 40% of all MDR cases circulating in the country, and

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the M strain by itself represents 40% of all XDR-TB cases. Both strains were found to carry *katG*315 mutation in the absence of mutation at *inhA*-15 in virtually all the isolates submitted to whole genome sequencing (Eldholm et al., 2015; Ritacco et al., 1997). Little attention has been paid to the genomic traits of other drug resistant strains circulating in Argentina. Our objectives were to analyze INH-resistant clinical isolates from Argentina (other than major strains M and Ra) for the presence of *katG*315 and *inhA*-15 mutations in relation to: [i] their genetic background, [ii] their ability to accumulate resistance to other drugs, and [iii] their ability to spread.

2. Materials and methods

2.1. Study population

Our laboratory acts as national reference laboratory for mycobacterial identification and drug susceptibility testing to first- and second-line anti-TB drugs. During the study period, January 2003-December 2012, a total of 7459 *M. tuberculosis* clinical isolates were referred to our laboratory from all over Argentina. Of these, 1797 were confirmed to be resistant to at least INH. In the design of the sample, we excluded isolates harboring M and Ra genotypes and repeated isolates from individual patients. When two or more isolates were available from a single patient, we included the one having the most extended drug resistance profile. Of the 1069 that were eligible, we selected one every three in chronological order, except for those harboring XDR patterns, which were all included in view of their relevance and small number. The resulting study sample consisted in 362 INH-resistant isolates (Fig. 1). Ethics committee approval and informed consent were not required because the work was retrospective and the data analyzed were anonymised.

2.2. Microbiological studies

The isolates were grown on Löwenstein-Jensen slants and identified as *M. tuberculosis* by biochemical and molecular tests. Drug susceptibility testing was performed by the reference standard proportion method in Löwenstein-Jensen medium and/or BACTEC MGIT 960 (Becton Dickinson, MD) under supranational proficiency testing according to World Health Organization standards (World Health Organization, 2009).

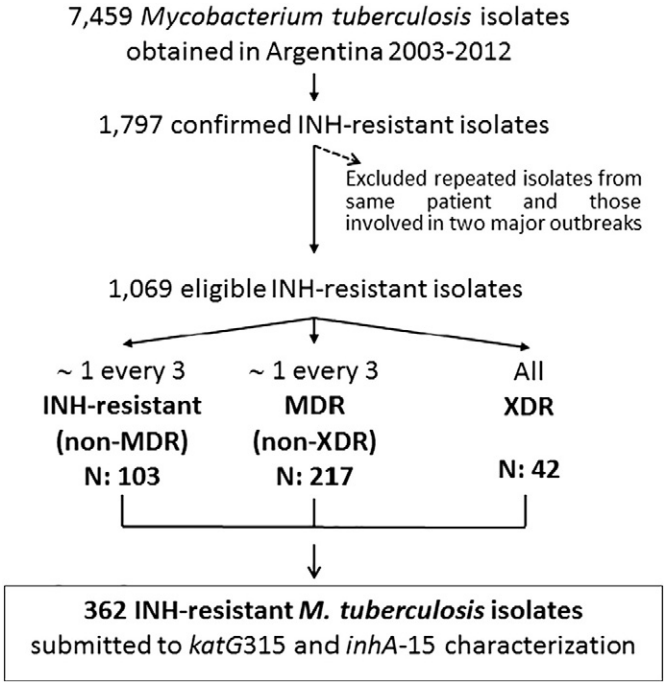

Fig. 1. Selection procedure for the 362 *M. tuberculosis* isolates included in the study.

2.3. Duplex allele-specific PCR for katG315 and inhA-15 characterization

An ad hoc modified version of a previously described multiplex allele-specific PCR was applied (Chia et al., 2012). The assay was adapted to detect mutation only at the canonical INH resistance sites, namely *katG*315 and *inhA*-15, using the following pairs of primers: [i] katG5R: *ATACGACCTCGATGCCGCT* and katG0F: *GCAGATGGGGCTGATC TACG*; and [ii] inhA-15: *CACCCCGACAACCTATCG* and inhAPF2: *GCGCGG TCAGTTCCACA*. The following reaction mix was used: 1X PCR reaction buffer; 4 mM $MgCl_2$; 1 mM dNTP mixture; 1 pM katG5R; 1 pM katG0F; 6 pM *inhA*-15; 6 pM inhAPF2; 0.5 U Taq Polymerase; DNA template (20 ng) and PCR-grade water to a final volume of 25 μl. Thermocycling consisted of an initial denaturation step at 96 °C for 3 min, 25 cycles of 95 °C for 50 sec, 68 °C for 40 sec, and 72 °C for 60 sec, and a final extension step at 72 °C for 7 min. The amplification patterns were examined by 3% agarose gel electrophoresis run in 1× TBE buffer. If the targeted location was wild type, the allele-specific fragments were amplified, yielding visible bands of 293 bp and 270 bp for *katG*315 and *inhA*-15, respectively. Any nucleotide substitution at the target location resulted in a missing band so that the mutation was incompletely characterized. A null-null pattern was considered suggestive of a double mutation. In order to control for amplification inhibition or other cause of technical failure, specimens yielding null-null patterns were submitted to the originally described multiplex allele-specific PCR, which is designed to screen for the two above-mentioned mutations plus the three *rpoB* mutations most frequently associated to rifampicin resistance.

2.4. Genotyping

We performed spoligotyping and RFLP IS*6110* according to international standard protocols (Kamerbeek et al., 1997; van Embden et al., 1993). We compared the spoligotypes in the SITVITWEB database available at the website of the Institute Pasteur Guadalupe (<http://www.pasteur-guadeloupe.fr:8081/SITVIT_ONLINE/>) (Demay et al., 2012. All isolates classified as belonging to the LAM family by spoligotyping were submitted to a multiplex PCR designed to identify the subtype LAM-RD^{rio}. The multiplex PCR was adapted from Gibson et al., 2008 as follow: 1X PCR reaction buffer; 1.5 mM $MgCl_2$; 2 mM dNTP mixture; DMSO 2.5 μl; 2 mM IS1561F: *GACCTGACGCCGCTGACAC*; 2 mM IS1561R: *CACCTACACCGCTTCCTGCC*; 4 mM RDRioBrgF: *CACTCCGGCTG CCAATCTCGTC*; 4 mM RDRioBrgR: *CACCGCCACGCTGAATGAGACCA*; 0.5 U Taq Polymerase; DNA template (20 ng) and PCR-grade water to a final volume of 25 μl. Thermocycling consisted of an initial denaturation step 5 min at 95 °C, followed by 45 cycles of 1 min at 95 °C, 1 min at 60 °C, and 4 min at 72 °C, with a final extension cycle of 10 min at 72 °C. As second marker we explored allelic diversity of MIRU loci 2 and 40 (Supply et al., 2006). A LAM isolate with two copies of MIRU2 and one copy of MIRU40 was considered to belong to the LAM-RD^{rio} type.

BioNumerics v5.1 (Applied Maths, St-Martens-Latem, Belgium) was used for clustering analysis using the unweighted pair group method with arithmetic averages. The isolates were considered to be in cluster when patterns showed 100% similarity in both RFLP and spoligotype. We tolerated differences either in the position/presence of one band in the RFLP IS*6110* or in the presence of one spacer in the spoligotyping.

2.5. Statistical analysis

We used MedCalc software v9.3.6.0 (Frank Schoonjans, Belgium) for statistical analysis. We used the χ^2 test for trend to analyze relationships between *katG*315 and *inhA*-15 polymorphisms with increasing drug resistance profiles and univariate analysis or Fisher exact test for relationships of *katG*315 and *inhA*-15 polymorphism with genotype and clustering. A P value <0.05 was defined as statistically significant and $P < 0.001$ as highly significant.

Table 1
Relationship between conspicuous spoligotype families or genotypes and two canonical isoniazid resistance mutations in 362 isoniazid-resistant *Mycobacterium tuberculosis* isolates, Argentina 2003-2012.

Family or genotype (N)	*katG*315MUT				*inhA*P-15 MUT			
	n	%	OR (95% CI)	*P*	n	%	OR (95% CI)	*P*
LAM (110)	86	78.2	4.9 (2.9–8.1)	<0.0001	11	10.0	0.2 (0.1–0.4)	<0.0001
Haarlem (59)	27	45.8	0.7 (0.4–1.2)	0.2052	20	33.9	1.4 (0.8–2.6)	0.2404
T1 Ghana* (45)	10	22.2	0.2 (0.1–0.4)	<0.0001	18	40.0	1.9 (1.0–3.6)	0.05
T1 Tuscany* (24)	2	8.3	0.1 (0.02–0.3)	0.0004	19	79.2	11.9 (4.3–32.8)	<0.0001
S (16)	4	25.0	0.3 (0.1–0.9)	0.0288	11	68.8	6.4 (2.2–18.8)	0.0008
Beijing (10)	8	80.0	3.6 (0.8–17.2)	0.1075	1	10.0	0.3 (0.04–2.2)	0.2353
X (7)	4	57.1	1.2 (0.3–5.3)	0.8377	1	14.3	0.4 (0.1–3.6)	0.44

MUT = mutated; wt = wild type; OR = odds ratio; CI = confidence interval. *The T family as a whole was not investigated because as such it is an ill-defined phylogenetic group.

3. Results

Among the 362 INH-resistant isolates, the frequencies of combined mutations were as follows: katG315MUT/inhA-15wt 192 (53.0%), katG315wt/inhA-15MUT 99 (27.4%), katG315wt/inhA-15wt 70 (19.3%), and katG315MUT/inhA-15MUT 1 (0.3%). The only double negative isolate in the study, which was XDR and resistant to ethionamide, was confirmed to harbor katG315MUT/inhA-15MUT, in addition to *rpoB*526 mutation by multiplex allele-specific PCR.

3.1. INH resistance mutation versus genotype

The frequencies of *M. tuberculosis* spoligotype families were: ill-defined T family 122 (33.7%), Latin-American & Mediterranean (LAM) 110 (30.4%), Haarlem 59 (16.3%), Unknown (U) 23 (6.4%), S 16 (4.4%), Beijing 10 (2.8%), and X 7 (1.9%). Orphan genotypes accounted for 15 (4.1%) isolates.

As for the genetic background of isolates of the LAM superfamily, the most frequently found in the study, the LAM-RD^{rio} signature was identified in only 14 isolates (12.7% of all LAM isolates). All 14 LAM-RD^{rio} isolates belonged to four spoligotypes (SIT42, SIT93, SIT177, and SIT469).

Mutations at *katG*315 and *inhA*-15 were unevenly distributed among genotypes. The LAM family was associated with katG315MUT/inhA-15wt. Isolates harboring LAM-RD^{rio} deletion did not differ significantly from LAM isolates lacking it regarding frequencies of the canonical mutations herein analyzed (Fisher Exact test, $P = 0.1822$). The S family was associated with katG315wt/inhA-15MUT (Table 1).

We did not search for association of the studied mutations with the T family as a whole because it is an ill-defined phylogenetic group. Instead, we searched for association with conspicuous genotypes within the T family. Only two SITs in this family showed significant association with the investigated polymorphisms. One is SIT53 T1 Ghana, which was positively associated with katG315wt/inhA-15wt (17/45, OR 3.0; 95% CI 1.5–5.9; $P = 0.0012$). The other is SIT159 T1 Tuscany, which was positively associated with *inhA*-15MUT and negatively associated with *katG*315MUT (Table 1). The remaining subfamilies and clades, including Haarlem 1 and Haarlem 3, were not associated with any of both mutations.

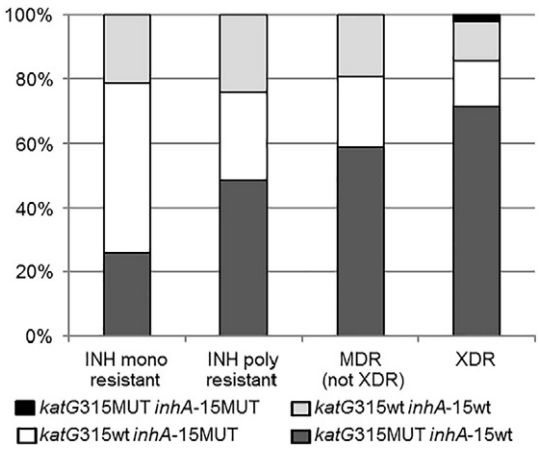

Fig. 2. Distribution of *katG*315 and *inhA*-15 polymorphisms across groups of isoniazid-resistant *Mycobacterium tuberculosis* isolates (n: 362) with increasing drug resistance profiles, Argentina 2003–2012. INH = isoniazid; MDR = multidrug resistant; XDR = extensively drug resistant; MUT = mutated; wt = wild type.

3.2. INH resistance mutations versus drug resistance profiles

For this analysis, the isolates were classified in four groups according to drug resistance profiles: [i] INH mono-resistant (n: 70), [ii] INH poly drug resistant (n: 33), [iii] MDR non-XDR (n: 217), and [iv] XDR (n: 42). Fig. 2 shows that the combination katG315MUT/inhA-15wt increased significantly across the groups (χ^2 test for trend 29.4; $P < 0.0001$). Conversely, the frequency of the combination katG315wt/inhA-15MUT showed a significantly decreasing trend (χ^2 test for trend 27.7; $P < 0.0001$). The frequency of isolates katG315wt/inhA-15wt did not vary significantly across the groups (χ^2 test for trend 1.2; $P = 0.27$).

3.3. INH resistance mutations versus clustering

Out of 304 isolates with available IS*6110* RFLP patterns, 204 (67.2%) were in cluster. *katG*315MUT was associated with clustering, *inhA*-15MUT showed no significant association, and the combination katG315wt/inhA-15wt was negatively associated with clustering (Table 2). Clustering frequencies did not differ significantly between LAM isolates harboring and lacking LAM-RD^{rio} deletion (Fisher Exact test $P = 0.4986$).

4. Discussion

We show here that *katG*315 is the most common INH resistance conferring mutation in Argentina, even after removing from the analysis the two leading outbreak strains harboring this mutation (Eldholm et al., 2015; Ritacco et al., 2012). Together, mutations at *katG*315 and *inhA*-15 covered about 80% of the isolates in our study, a percentage that is in line with global rates (Seifert et al., 2015). In Supplementary Table 1, we compared frequencies of both mutations in our study with

Table 2
Relationship between combinations of isoniazid resistance mutations and clustering in 304 INH-resistant *Mycobacterium tuberculosis* isolates with available RFLP *IS*6110 patterns, Argentina 2003–2012.

Mutation combination	N	In cluster		OR (95% CI)	*P*
		n	%		
*katG*315MUT *inhA*-15wt	178	132	74.2	2.1 (1.3–3.4)	0.0035
*katG*315wt *inhA*-15MUT	70	50	71.4	1.3 (0.7–2.3)	0.4296
*katG*315wt *inhA*-15wt	55	22	40.0	0.2 (0.1–0.4)	<0.0001
*katG*315MUT *inhA*-15MUT	1	-	-	-	-

MUT = mutated; wt = wild type; OR = odds ratio; CI = confidence interval.

frequencies reported in relevant studies from the Americas, Europe and Africa, many of which were included in a recent systematic review (Seifert et al., 2015). Therein we showed that: [i] *katG*315MUT concurs only exceptionally with *inhA*-15MUT in Argentina, [ii] this negative association has numerous antecedents in the literature but is not equally conspicuous globally, and [iii] even when detected, this negative association has not been sufficiently emphasized.

A lucid early work had already called attention to the negative association between *katG*315MUT and *inhA*-15MUT even before the role of mutation at the *inhA* promoter was fully acknowledged as a mechanism for INH resistance (Musser et al., 1996). More recently, other studies have also explicitly remarked it (Hazbón et al., 2006; Schaaf et al., 2009). Hazbón et al. (2006) postulated that during strain evolution the double mutation might be counter-selected because of fitness loss. The finding of only one isolate harboring the double mutation in our study supports this hypothesis. Indeed, we were not able to detect a secondary case or any match to this IS*6110* RFLP pattern in the national *M. tuberculosis* genotype database, which contains over 7000 patterns of isolates obtained between 1992 and 2012.

4.1. INH resistance mutation versus genotype

Published data suggest that strains with different genetic backgrounds differ in their ability to acquire drug resistance and cause disease, being Beijing strains the most conspicuous example (Fenner et al., 2012). However, this remains a controversial issue because lineage-specific factors are not easy to assess (Eldholm and Balloux, 2016). In Argentina, Beijing strains do exist, but at very low rates (Ritacco et al., 2008). In line with the country historical and ethnic background, strains of the Euro-American lineage are widely dominant in the *M. tuberculosis* overall population. To this lineage belong the two MDR strains driving the longstanding MDR-TB epidemic in Argentina. We show here that, as expected, Euro-American strains also predominate among less successful drug resistant strains circulating in the country. At the sublineage level, we find LAM strains, but not Haarlem strains, to be strongly associated with *katG*315MUT. These results are not totally congruous with results of previous studies from South American countries, suggesting a heterogeneous distribution in the affinities for these mutations (Dalla Costa et al., 2009, de Freitas et al., 2014).

It has been proposed, but remains controversial, that the LAM-RD^{rio} type has an increased dissemination rate and a biological advantage. This type has also been associated with INH resistance and MDR status in Brazil and elsewhere (Barbosa Cde et al., 2012, Dalla Costa et al., 2013, Lazzarini et al., 2007, Von Groll et al., 2010, Weisenberg et al., 2012). Herein we find that in Argentina LAM strains harboring RD^{rio} deletion: (i) do not contribute substantially to the burden of drug resistant TB, and (ii) do not differ from RD^{rio} wild type LAM strains regarding clustering and type of INH conferring mutation. Furthermore, strain Ra, the most conspicuous MDR LAM strain in Argentina, does not harbor the LAM-RD^{rio} deletion (data not shown).

As for other genotypes, we found that INH-resistant strains of the S family and the T1 Tuscany clade preferentially harbor *inhA*-15MUT whereas isolates of the T1 Ghana clade preferentially harbor the combination katG315wt/inhA-15wt. Altogether, these results reinforce the idea that certain genotypes are prone to select particular drug resistance mechanisms, as suggested by Torres et al., 2015 for the Indo-Oceanic group.

4.2. INH resistance mutations versus drug resistance profiles

In our study, both an increasing frequency of *katG*315MUT and a concomitant decreasing frequency of *inhA*-15MUT correlated tightly with increasingly broad drug resistance profiles. Some studies found *katG*315MUT to be associated with resistance to streptomycin, ethambutol, or both (Hazbón et al., 2006, Huyen et al., 2013, van Doorn et al., 2006) and, with a single exception (Baker et al., 2005), there is consensus in acknowledging an association of *katG*315MUT with multidrug resistance (Fenner et al., 2012, Hazbón et al., 2006, Huyen et al., 2013, van Soolingen et al., 2000).

Thus, in many settings including ours, INH-resistant isolates harboring *katG*315MUT seem to be able to accumulate further drug resistance mutations and, therefore, to evolve more easily towards MDR and XDR status than *katG*315wt INH-resistant isolates. Our results also suggest that, in Argentina, INH-resistant strains harboring *inhA*-15MUT are less prone to accumulate resistance to other drugs and that katG315wt/inhA-15wt strains are equally likely to acquire or not further drug resistance. However, this is not the case for other settings – like Lisbon in Portugal and the Western and the Eastern Cape Provinces in South Africa – where the predominant MDR- and XDR-TB clones were found to harbor *inhA*-15MUT (Machado et al., 2013, Müller et al., 2011).

4.3. INH resistance mutations versus clustering

Regarding transmission, we found *katG*315 mutation to be strongly associated with clustering, what is consistent with results of previous studies from Europe and the USA (Fenner et al., 2012, Gagneux et al., 2006, van Doorn et al., 2006). In a recent study, Jagielski et al., 2015 suggested that *katG*315MUT strains have a relatively low transmissibility because more than two-thirds of the cases with *katG*315MUT isolates in their study were not in cluster (Jagielski et al., 2015). Our results, however, do not endorse their view, because 74% of our *katG*315MUT isolates were indeed in cluster, reinforcing the idea of this mutation being associated with disease transmission.

Some authors pointed out that *inhA*-15MUT strains were also able to spread in the community (Fenner et al., 2012, Gagneux et al., 2006). Our present findings support these observations because strains of the Tuscany clade harboring *inhA*-15MUT were indeed included in a conspicuous cluster in Argentina representing near 5% of all newly diagnosed MDR cases in the country. Although they are not highly frequent or prone to accumulate further drug resistance, strains of this particular clade are still able to disseminate and linger in the community (Palmero et al., 2005, Ritacco et al., 2012).

4.4. Limitations

This work has two major limitations. First, owing to budget and operational reasons, the study sample was designed to cover only one third of INH-resistant isolates referred to our laboratory in the period. However, we selected the isolates at random to represent INH-resistant TB cases occurred countrywide and losses were expected to be evenly distributed among clades. Missing isolated may have mainly affected clustering analysis by reducing the number of observed clusters, without necessarily biasing the associations reported. Second, in Argentina, drug susceptibility testing is provided only to cases at risk of drug resistance (TB treatment failure, default or relapse, exposure to a drug-resistant TB case, infection with HIV or other immunosuppressing condition). Thus, it is possible that strains with low INH resistance did not arrive to our reference laboratory because the evolution of the patients was good. In any case, we could still assume that our results expose features of the most relevant strains in clinical and epidemiological terms. Third, we did not investigate all known INH resistance conferring mutations. Particularly for the two gene sites analyzed, we did not further assess the nucleotide change involved, which would have certainly enriched the analysis.

5. Conclusions

In Argentina, *katG*315 is the most common INH resistance conferring mutation site and rarely coexists with *inhA*-15 mutation. Isolates carrying *katG*315MUT tend to accumulate resistance to other drugs and also to be transmitted. Isolates harboring *inhA*-15MUT are less likely to be in cluster and to develop further drug resistance. INH-resistant strains carrying *katG*315wt/*inhA*-15wt are most frequently found as orphan

isolates, indicating that INH resistance mechanisms other than *katG*315MUT and *inhA*-15MUT impair epidemiological fitness. Certain genotypes are prone to preferentially harbor one, the other, or none of the investigated INH resistance mutations.

We conclude that in Argentina, *M. tuberculosis* strains with particular genetic backgrounds select for alternative INH resistance genetic mechanism and differ between each other in the ability to spread and the ability to accumulate resistance to other drugs. Although epidemiological and host factors could also be modulating these outcomes, our findings support the systematic genotyping of drug resistant clinical isolates to identify suspects of harboring INH resistance mutations more associated to transmission or to the acquisition of additional resistances. However, our observations cannot be extended to any setting without a robust knowledge about drug resistant associated mutations and molecular epidemiology of the circulating strains.

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Transparency declarations

None to declare. Conceived and designed the experiments: VR. Performed the experiments: JM CL. Wrote the manuscript: JM VR. Analyzed the data, read and approved final version: JM RP CL NS KY LD BL VR.

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