Tuberculosis 92 (2012) 56–59

Contents lists available at SciVerse ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube

MOLECULAR ASPECTS

Characteristics of multidrug-resistant *Mycobacterium tuberculosis* in southern Brazil

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ARTICLE INFO

Article history:
Received 14 June 2011
Received in revised form
8 September 2011
Accepted 20 September 2011

Keywords:
Tuberculosis
Molecular epidemiology
Multidrug-resistance
Brazil

SUMMARY

A major threat to tuberculosis (TB) control programs is the emergence of drug resistant *Mycobacterium tuberculosis* strains that cause TB that cannot be cured by standard anti-TB drug regimens. Because few data exist on MDR-TB in this region of the country, we performed an epidemiologic study that combined conventional and molecular analysis of MDR-TB cases from Rio Grande do Sul (RS) that were diagnosed in this period and included cases that were under treatment with second line drug schemes. Included were 121 MDR cases and sequencing of *rpo*B and *kat*G showed that 106 (87.6%) strains were mutated in *rpo*B and 97 (80.2%) in *kat*G. Spoligotyping demonstrated that the LAM genotype was predominant ($n = 70$, 57.8%) and included the largest group composed by 22 (18.1%) strains with the LAM5 ST93 genotype. Other main genotypes belonged to the families T ($n = 22$, 18.2%), U family ($n = 16$, 13.2%), Haarlem ($n = 5$, 4.1%) and X ($n = 1$, 0.8%). Genotyping by IS*6110*-RFLP analysis showed 51 distinct fingerprints, 38 (31.4%) of these observed only once and the other 13 patterns being shared among the rest of the isolates ($n = 83$, 68.6%). Among the 22 strains that were LAM5 ST93, only two had different IS*6110*-RFLP genotypes. In conclusion, there exists a high degree of *M. Tuberculosis* genotype clustering among MDR-TB cases in Rio Grande do Sul. Moreover, we observed a large MDR-TB outbreak.

1. Introduction

Besides co-infection of TB patients with HIV/AIDS, a major threat to TB control programs is the emergence of drug resistant (DR) and multidrug-resistant (MDR) *Mycobacterium tuberculosis* strains that cannot be cured by standard anti-TB drug regimens.[1,2] Resistance to at least the two major anti-tuberculosis drugs, rifampin (R) and isoniazid (H), is termed MDR and treatment of such cases requires prolonged and expensive chemotherapy.[3] The State of Rio Grande do Sul (RS) is located in southern Brazil and according to its Secretary of Health (SES/RS),[4] in the period of 2004–2006, 718 new TB cases including 51 cases of MDR-TB were reported, with an incidence of respectively drug susceptible and resistant disease of 51.3 and 3.6/100,000. The aim of this study was to characterize MDR-TB cases from RS, emphasizing the molecular epidemiology of strain during three-years of strain and data collection.

2. Materials and methods

2.1. Study setting

The 121 MDR strains were from "Hospital Sanatório Partenon" (HSP) and the "Laboratório Central do Rio Grande do Sul" (LACEN/RS), both being reference institutions for TB diagnosis in the State. Isolates of 51 new MDR-TB cases from 2004 to 2006 and from 70 cases of treatment abandon or failure before the period of the study. These 70 patients were submitted to MDR-scheme therapy used in Brazil until 2010 that included streptomycin (S), ethambutol

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doi:10.1016/j.tube.2011.09.008

(E), pirazinamid (Z) and ethionamide, keeping positive culture between 2004 and 2006. This study was approved by the ethical committee of the School of Public Health of Rio Grande do Sul (protocol CEP/ESP-164/2009).

2.2. Susceptibility test

Susceptibility test was performed as described by Canetti et al. (1969)[5] on isolates at LACEN/RS and the drugs used are H, R, EMB and S, respectively.

2.3. Molecular analysis

Chromosomal DNA was extracted from cultures on L-J medium, using the CTAB method.[6] Spoligotyping was performed according to Kamerbeek et al. (1997).[7] IS*6110*-RFLP typing was performed as described by Van Embden.[6] Mutations in part of the *kat*G and *rpo*B genes were sequencing according to Silva et al. (2003).[8,9]

2.4. Statistical analyses

Epi-Info (version 3.5.1, Center for Disease Control & Prevention, Atlanta, USA) was used for statistical analyses.

3. Results

3.1. Patient characteristics

Among the patients, 58.6% were male between 12 and 69 years old; the 41.3% females ranged between 18 and 77 years of age. Serological tests were made in 90 (74%) patients to check the positivity of HIV and 25 (27.7%) were serum positive; 15 of them were male (60%) and 10 (40%) female.

3.2. Resistance profile in MDR strains

All isolates were considered MDR by results of phenotypic tests and among these, 88 (72.7%) cases had resistance for R and H only. The other strains presented resistance to an additional drug, being S in 18 cases (14.8%), EMB in three cases (2.4%), Z in four cases (3.3%) and S + EMB in one case. Furthermore, between the 25 cases of co-infection MDR-TB/HIV, there were 20 (80%) with resistance for RH, three cases (12%) with resistance for RH + S and finally two cases (8%) with RH + EMB resistance.

3.3. Sequencing of katG and rpoB genes

In *rpo*B gene, mutations were observed in 106 isolates (87.6%), the majority ($n = 70$, 57.9%) in codon 531 and all but two of these isolates presenting a change of the amino acid serine (TCG) to lysine (TTG). SNPs in other regions were observed, mostly in codon 526. The *kat*G gene was mutated in 97 (80.2%) of the isolates, predominantly ($n = 95$, 78.5%) in codon 315, all being a substitution from AGC to ACC, leading to the amino acid change of serine to threonine. In addition, an SNP was identified in both codons 297 and 299, causing the change of glycine (GGC) to aspartic acid (GTC) and glycine (GGC) to serine (AGC). Wildtype (WT) alleles of *kat*G were observed in 24 (19.8%) isolates; and there were 15 (12.4%) WT isolates for *rpo*B. Seven isolates (5.8%) presented WT alleles for both genes.

3.4. Spoligotyping analysis

This fingerprinting technique demonstrated the existence of 34 different genotypes among the MDR-TB isolates, 19 being unique patterns (15.7%) and the remaining 15 patterns being shared among 102 (84.3%) of the isolates. Upon analysis of spoligotype classification as defined by Brudney et al.,[10] we observed that clusters were composed respectively of H1 (ST 47), LAM1 (ST 729), LAM 1 (ST 20), LAM2 (ST17), LAM3 (ST33), LAM4 (ST 60), LAM5 (ST93), LAM6 (ST64), LAM9 (ST177), LAM9 (ST 42), T1 (ST 53), T1 (ST 65), T5_MAD2 (ST 58), U (ST 863), U (ST 106), X2 (ST137) and X3 (ST92). Six of the spoligopatterns (5.8%) had not been reported before, thus, all being orphan patterns. More than half of the isolates ($n = 70$, 57.8%) belonged to the LAM family, 22 (18.2%) were of the T family, 16 (13.2%) of the U family, five isolates (4.1%) presented a Haarlem genotype and two isolates (1.65%) belonged to the X family. The major cluster ($n = 22$; 18.2%) was composed of samples with the LAM5 genotype (ST93).

Table 1
Cluster analysis by using two techniques of strains typing.

Technique	*n* of unique strains	*n* of strains in cluster	*n* of clusters
Spoligotyping	19	102	15
IS*6110*-RFLP analysis	38	83	13
Spoligotyping + IS*6110*-RFLP	40	81	13

3.5. RFLP analysis

All 121 MDR-isolates yielded RFLP patterns with the number of IS*6110* copies ranging from four to 15 copies and presented a mean copy number of 10 bands. We observed less than six IS copies among six isolates, three of these with four bands and three with five IS copies. A total of 51 distinct fingerprints were observed and 38 (74.5%) of them were unique, while the other 13 patterns comprised 83 (68.6%) isolates, with cluster size ranging from two to 20 isolates. Combined spoligotyping and RFLP analysis showed that 81 isolates kept belonging to some cluster (Table 1).

Of particular interest was the biggest cluster composed of 22 isolates of the LAM5-ST93 spoligotype, which presented three RFLP patterns, one with 20 isolates and two others belonging to a single pattern each. This cluster included two of the 11 isolates from cases of primary resistance.

3.6. Lineages and mutations

The LAM lineage appears as the major family, with 57.8% of the studied samples, and presented five WT strains at *rpo*B gene and 16 WT at *kat*G. It is also meaningful that among the 11 cases of primary resistance, all belonged to the LAM family and carried *kat*G and *rpo*B mutation (Table 2). No WT strains had the U genotype. T and

Table 2
Mutations on cases of primary resistance.

STs[a]	Subfamily	*kat*G mutation	*rpo*B mutation
20	LAM1	315 AGC-ACC S-T	WT[b]
33	LAM3	315 AGC-ACC S-T	526 CAC-TAC H-Y
42	LAM9	315 AGC-ACC S-T	526 CAC-TAC H-Y
42	LAM9	315 AGC-ACC S-T	531 TCG-TTG S-L
42	LAM9	315 AGC-ACC S-T	531 TCG-TTG S-L
93	LAM5	315 AGC-ACC S-T	531 TCG-TTG S-L
93	LAM5	315 AGC-ACC S-T	531 TCG-TTG S-L
211	LAM3	WT	531 TCG-TTG S-L
Unknown	–	315 AGC-ACC S-T	526 CAC-CCC H-P
Unknown	–	315 AGC-ACC S-T	531 TCG-TTG S-L
Unknown	–	WT	WT

[a] Shared types.
[b] Wild type.

Haarlem families presented mutations in all but two and one strain, respectively. Among the strains of the majo cluster with 22 samples (LAM5 ST93), 20 strains presented mutation in *kat*G and 21 in *rpo*B (Table 2). One isolate belonging to this cluster was determined as WT for both genes and interestingly, one of the two isolates with no mutation at *kat*G gene had different RFLP pattern.

4. Discussion

This study relates the biodiversity of a selection of MDR-*M. tuberculosis* isolates as determined by genotyping. Among the 121 isolates genotyped by RFLP, 51 different patterns were identified, whereas 34 distinct types were observed by spoligotyping. The strains presented 13 clusters upon RFLP analysisand 15 clusters after spoligotyping, presenting high clustering rates of respectively 68.6% and 84.3%, confirming earlier studies showing that IS*6110*-RFLP analysis is more discriminative than spoligotyping for genotyping *M. tuberculosis* isolates in Brazil.[11,12] The very high level of clustering observed in both genotype procedures was surprising and may be related with the fact that the State of Rio Grande do Sul has one of the highest incidences of TB in Brazil: 48/100.000[4]. In addition, there exist neighbourhoods in the metropolitan area of Porto Alegre presenting higher disease incidence and transmission rates, and the city of Porto Alegre, the State capital, presents an incidence of 93/100.000, the highest in Brazil.[4]

The biggest cluster in the present study is composed by 22 isolates that share the LAM5-SIT93 genotype. Interestingly, 20 of these isolates also presented identical RFLP types, confirming the genetic homogeneity of this group, while the other two isolates had particular RFLP types and did not share the patterns with strains of the study. Interestingly, one of these unique patterns differs with a resistance for RH + S, and had no mutation at *kat*G gene. Nonetheless, this lineage may be particularly efficient to generate SNPs that configure resistance profiles. Both the high cluster level observed and the existence of a single genotype among 15% of the MDR isolates alerts for the need of further investigation of adequacy of the TB control program in Porto Alegre, in the way this cluster had been infected people during three years at least.

The second most frequent were strains with the T genotype (18.8%), followed by strains with the spoligotype characteristic for the U family. This is also observed in studies on drug susceptible TB in patients from RS, however, as we could see, less strains of the Haarlem type (4%) seem to be present in the drug resistant strain population, when compared to studies in the State of RS with susceptible strain population.[13,14] The remaining genotypes contributed to only 10.7% of all isolates. In general, this adds more data to confirm that the most prevalent *M. tuberculosis* genotype also among the MDR strains is that of the LAM family (58.8%).

Spolitotypes as LAM2 ST17, LAM9 ST42, LAM5 ST93 and U ST106 were the most frequent. Among the 10 strains with LAM2 ST17, five of them had the same mutation in *rpo*B gene (531 TCG-TTG S-L) and shared a single RFLP pattern. Twelve strains were LAM9 ST42 and seven of them had the same mutations in both *kat*G and *rpo*B genes; however, only two isolates shared the same patterns in RFLP. U family ST93 had six isolates and all of them had the same mutations at *kat*G and *rpo*B (315 AGC-ACC S-T and 531 TCG-TTG S-K, respectively); four of them shared same RFLP pattern.

Interesting was our observation that the largest cluster including 15% of strains of the study was of the LAM5 type, which is barely observed among drug susceptible strains in studies of the same region of Brazil.[13,15] The LAM5 ST93 cluster included a single WT strain while all the others presented 315 AGC-ACC S-T mutation on *kat*G; all the strains had 531 TCG-TTG S-L mutation on *rpo*B gene.

Accordingly to Pym et al.,[16] isolates with the Haarlem genotype are quite frequent in TB cases in Brazil, but not frequently observed in the present study (4%; including SIT47 and SIT50) or in other genotype studies in the region.[11,17] The presence of the 315-*kat*G mutation has been associated with the Haarlem genotype and resistance to isoniazid in another study in this region of Brazil[15] and this was confirmed here, observing this SNP in four of the five Haarlem strains.

Most of the *rpo*B gene SNPs occurred at codon 531 ($n = 70$; 57.8%), causing the amino acid change from Serine (TCG) to Lisine (TTG), also in accordance with literature data.[18] The mutation in codon 315 of the *kat*G gene leading to the amino acid change Serine (AGC) to Threonine (ACC) was observed in 97.9% of the *kat*G mutated strains, also in concordance with earlier data.[15] We also observed an insertion of 12 nucleotides (CCAGAACAACCC) at codon 516 in *rpo*B gene that had not been described previously, in three strains, one with the LAM4 ST60 genotype and two more with U family ST863 with no RFLP correlation.

In conclusion, we observed that MDR-TB in RS is caused mostly by strains that had been transmitted in the last few years, as demonstrated by the large level of genotype clustering and the existence of some large clusters. The predominance of the LAM5 genotype among strains in a general TB population that is mostly LAM but not infected by this particular genotype is suggestive for an MDR specific outbreak. This suggests that genotyping could predict the spreading of MDR strains in part of the cases and could be useful to improve the management of TB in this region of Brazil.

Acknowledgements

Many thanks for CNPq, CDCT/FEPPS and Laboratory of Molecular Biology Applied to Mycobacteria at IOC/FIOCRUZ for the financial support and infrastructure.

Funding: None.

Competing interest : None Declared.

Ethical approval: Not required.

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