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Research Paper

Mycobacterium tuberculosis belonging to family LAM and sublineage RD^{Rio}: common strains in Southern Brazil for over 10 years

Renata Oliveira Soares, Maíra Bidart de Macedo, Andrea von Groll, Pedro Eduardo Almeida da Silva

Universidade Federal do Rio Grande, Rio Grande, RS, Brazil.

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Abstract

A sublineage of *Mycobacterium tuberculosis* called RD^{Rio} was described in 2007. Although only recently described, this strain may have been present previously in the population, and its identification in clinical isolates will elucidate bacterial transmission dynamics and host-pathogen interactions. This study evaluated the clonal diversity of the RD^{Rio} sublineage in clinical isolates from Rio Grande-RS obtained between 1998 and 2001. Among the 45 samples analyzed by the MIRU-VNTR method, there were six clusters with two samples each and 33 orphan strains with unique pattern. The strains were distributed across several different lineages including LAM (34.04%), X (14.89%), Haarlem (12.77%), UgandaI (10.64%), S (4.26%), NEW-1 (2.13%) and Cameroon (2.13%); 14.89% of the strains matched to multiple lineages. RD^{Rio} strains were present in 28.9% of the samples and 81.25% of the identified strains belonged to the LAM family. The high clonal diversity observed in this study is a constant feature in this region. The RD^{Rio} sublineage has been in Rio Grande-RS since 1998. The continued monitoring of RD^{Rio} in clinical isolates will enhance the understanding of its epidemiological significance.

Key words: *Mycobacterium tuberculosis*, MIRU-VNTR, RDRio.

Introduction

Tuberculosis (TB) is a global public health problem. There were 9.27 million new TB cases in 2007 with an incidence of 139 cases per 100,000 people (World Health Organization, 2009). Brazil, with 72,000 new cases reported annually, is among the 22 countries that account for 80% of the TB cases worldwide (World Health Organization, 2009). Rio Grande city, located in southern Rio Grande do Sul, has an incidence of approximately 70/100,000 within the municipalities prioritized for TB control (Ministério Da Saúde Do Brasil, 2010).

Several molecular biology tools have become available to support TB management. For instance, *Mycobacterium tuberculosis* genotyping determines the geographical and spatial distribution of strains, the predominance of different genotypes and potential bacterial adaptations to specific human populations (Brudey *et al.*, 2006; Filliol *et al.*, 2006; Gagneux *et al.*, 2006). Genotyping assays using MIRU-VNTR (*Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat*) amplify independent loci and quantify the repeat regions at each locus (Viedma *et al.*, 2005). Repeat pattern similarities are used to classify new isolates into clades, genotype families and/or strains (Supply *et al.*, 2001, 2003).

Lineage identification with MIRU-VNTR is facilitated by MIRU-VNTRplus database, which contains genotyping data from a reference collection of 186 isolates representing the main *M. tuberculosis* lineages. This collection includes *M. tuberculosis* strains of the following lineages: W/Beijing, Cameroon, Delhi/Central Asian, East African-Indian, Ghana, Haarlem, Latin American-Mediterranean (LAM), Turkish, S, Uganda I and II, Ural, and X (Allix-Béguec *et al.*, 2008; Supply *et al.*, 2006).

Recently, the predominant genotype of *M. tuberculosis* in Rio de Janeiro, Brazil, was identified (Lazzarini *et al.*, 2007). This sublineage, RD^{Rio}, is a derivative of the LAM lineage and has a deletion of ten genes (26.317 kb), or approximately 0.6% of the genome (Cole 2002; Lazzarini *et*

Send correspondence to P.E.A. Silva. Universidade Federal do Rio Grande, Rio Grande, RS, Brazil. E-mail: pedrefurg@gmail.com.

al., 2007). RD^{Rio} strains may have higher transmission rates and could cause more severe TB (Lazzarini *et al.*, 2008). In this study, the RD^{Rio} strains in isolates from Rio Grande between 1998 and 2001 were characterized, and the major lineages present in the population were studied. Despite being only recently described, RD^{Rio} strains could possibly have been circulating for much longer, and its identification in older clinical isolates may lead to a better understanding of its transmission dynamics and interactions with its host.

Materials and Methods

Samples

Forty-five *M. tuberculosis* strains were isolated from patients admitted to the University Hospital of Rio Grande, RS, Brazil, between 1998 and 2001. These strains are archived in the Mycobacteriology Laboratory, Faculty of Medicine at Federal University of Rio Grande.

Genomic DNA extraction

Each bacterial pellet was transferred to a microtube and resuspended in 300 µL of Tris-EDTA (TE). The bacteria were inactivated by heating at 80°C for 30 min. The microtube was centrifuged at 5000 *g* for 5 min, and the supernatant containing the DNA was removed. The extraction products were stored at -20°C.

MIRU-VNTR genotyping

The MIRU-VNTR 12 loci method was performed according to Supply *et al.* (2000). The results from PCR of each locus (2, 4, 40,10, 16, 20, 23, 24, 26, 27, 31 and 40) were combined to form a 12-digit allelic profile that was used to analyze the genetic relationships.

Strain identification was performed with tools on the MIRU-VNTRplus website (http://www.miru-vntrplus.org). The distance between each locus was compared with the data from 186 strains in the website reference collection. While this distance is adjustable, we initially selected 0.17, which corresponds to four loci of difference tolerance when MIRU-VNTR is the only method used (Allix-Béguec *et al.*, 2008). Similar strains were grouped, and based on the database reference sequences, strains in the samples of interest were identified.

Analysis of genetic relationships

Dendrograms were constructed with the tools on the MIRU-VNTRplus website (Allix-Béguec *et al.*, 2008), and clusters were grouped by UPGMA (*Unweighted Pair Group Method* with *Arithmetic Mean*). Clusters were defined with at least two strains of *M. tuberculosis* isolates with identical patterns in different patients. The discriminatory power of MIRU-VNTR was calculated using the discriminatory index of Hunter-Gaston (HGDI). The HGDI was calculated by the following equation: $HGDI = 1 - [1/N(N-1) \Sigma n_j (n_j-1)]$ with $j = 1$, where N is the total number of strains, is the total number of different patterns and n_j is the number of strains belonging to each pattern (Hunter and Gaston, 1988). The allelic diversity (h) of MIRU-VNTR in each of the 12 loci was calculated by the equation $h = 1 - \Sigma xi^2 [n/(n - 1)]$, where xi is the allele frequency at each locus and n is the number isolates (Selander *et al.*, 2006).

Identification of the RD^{Rio} lineage strains

The identification of RDRio lineage strains was performed with a PCR protocol modified as previously described (Gibson *et al.*, 2008). PCRs were performed in 30 µL containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 400 mM of each primer (RDRioBrg and IS1561), 200 mM deoxyribonucleoside triphosphates (Invitrogen™), 6% glycerol, 1 U Taq DNA polymerase (Invitrogen™) and 1.5 µL of isolate DNA.

DNA amplifications were performed under the following conditions: 1 cycle at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min; and 1 cycle at 72 °C for 10 min. PCR products were electrophoresed on a 1.5% agarose gel for 1 hour at 90 V, and the presence of either a 1175 bp band, corresponding to an RDRio strain, or a 530 bp band, corresponding to non-RDRio strains, was observed.

Results and Discussion

MIRU-VNTR

Thirty-nine different MIRU-VNTR patterns were detected in the 45 strains analyzed. Twelve strains were grouped into six clusters of two samples each, and 33 strains had unique patterns (Figure 1).

The analysis of allelic diversity at each locus (Table 1) showed that loci 10, 16 and 40 were highly discriminatory ($h \geq 0.6$), while loci 23, 26, 27 and 31 were moderately discriminatory ($h \geq 0.3$). Loci 2, 4, 20, 24 and 39 had low discriminatory power ($h < 0.3$). These results are similar to those in multiple previous studies (1, 9, 17, 19, 21). As noted by Sola *et al.* (2003), there is a hierarchy of polymorphism, such that loci 10, 23, 26, 31 and 40 have greater discriminatory power than the others.

The MIRU-VNTR 12 loci method is quick and easy, and it has been evaluated in several studies in different locations (Kang *et al.*, 2009). Because it has a discriminatory power equivalent to RFLP (Restriction Fragment Length Polymorphism), this method is an alternative to IS6110-RFLP for epidemiological studies (Mazars *et al.*, 2001; Supply *et al.*, 2000). As in other studies, we found that the HGDI of MIRU-VNTR was 0.994, indicating a high discriminatory power (Sun *et al.*, 2004; Silva *et al.*, 2009; Von Groll *et al.*, 2010).

This technique has limitations, and it could be combined with an additional genotyping method for greater ac-

curacy (Cowan *et al.*, 2005; Van Deutekom *et al.*, 2005; Kang *et al.*, 2009). Different combinations of MIRU and other loci have been evaluated. In future studies, MIRU-VNTR could be standardized to include 15 loci for epidemiological studies and 24 loci for phylogenetic studies of *M. tuberculosis* (Supply *et al.*, 2006).

The strains were distributed among the following lineages: LAM (16 strains, 34.04% of the total); X (7,

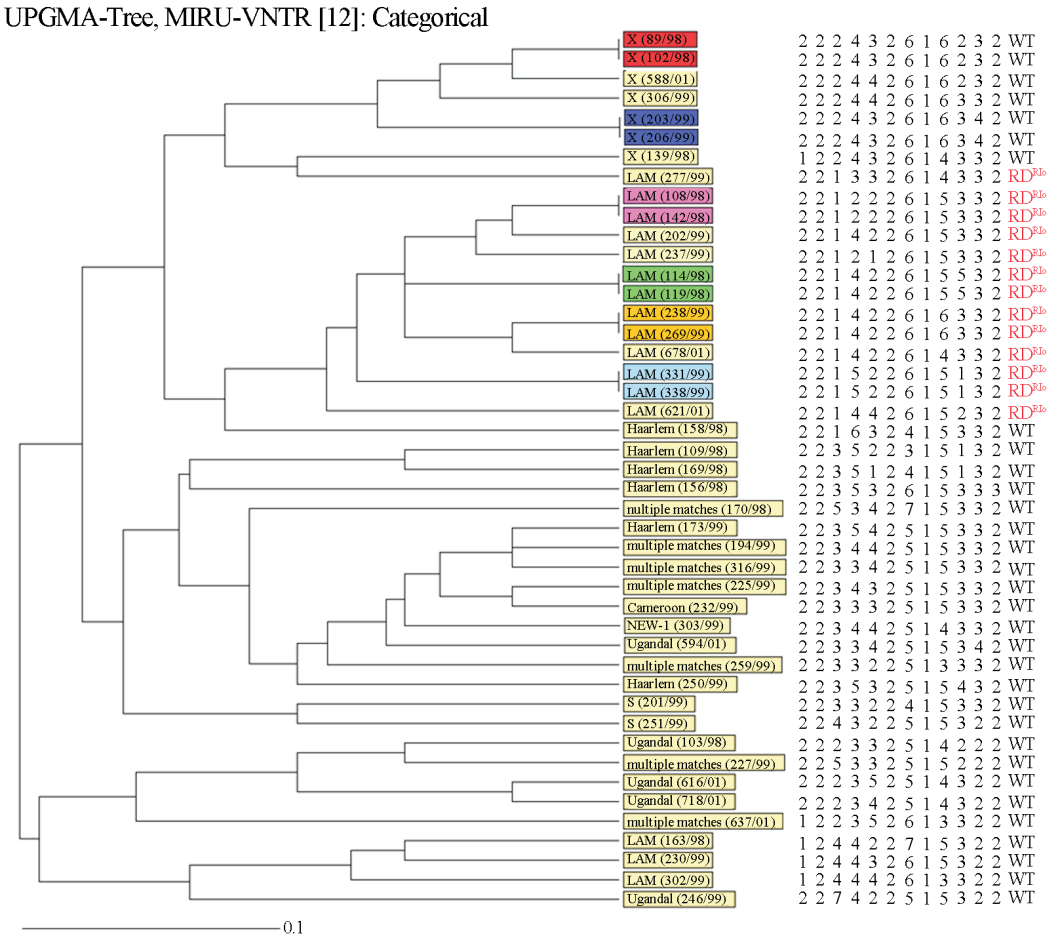

Figure 1 - Dendrogram of genetic relationships between 45 isolates of *M. tuberculosis*, as determined by MIRU-VNTR 12 loci analysis.

Table 1 - MIRU-VNTR Allelic distribution in 45 isolates

Variation lélica	Locus											
	2	4	10	16	20	23	24	26	27	31	39	40
0	0	0	0	0	0	0	0	0	0	0	0	0
1	5	0	0	2	0	0	45	0	4	0	0	14
2	40	43	3	16	45	0	0	0	6	10	44	11
3	0	2	13	14	0	1	0	3	32	32	1	13
4	0	0	21	11	0	3	0	7	1	3	0	4
5	0	0	7	2	0	15	0	27	2	0	0	2
6	0	0	1	0	0	24	0	8	0	0	0	0
7	0	0	0	0	0	2	0	0	0	0	0	1
Allelic diversity (h)[a]	0.18	0.06	0.66	0.71	0	0.59	0	0.57	0.45	0.43	0.02	0.74
ID[b]	Low	Low	High	High	Low	Mod.	Low	Mod.	Mod.	Mod.	Low	High

[a]$h = 1 - \Sigma\, xi^2\, [n/(n-1)]$.
[b]The power of Discriminatory Index is defined as: High (≥ 0.6), Moderate (< 0.6 and ≥ 0.3) and Low (< 0.3).

14.89%); Haarlem (6, 12.77%); Uganda I (5, 10.64%); S (2, 4.26%); NEW-1 (1, 2.13%); and Cameroon (1, 2.13%). Seven strains (14.89%) were matches to multiple lineages (*i.e.*, more than one family was within the same phylogenetic distance).

Identification of RD^{Rio} strains

Of the 45 strains analyzed, 13 (28.9%) belonged to the RD^{Rio} lineage, and the other 32 strains were grouped as non-RD^{Rio}. In previous studies, the RD^{Rio} lineage comprised 30% of all isolates in Rio de Janeiro (Lazzarini *et al.*, 2007), 37% in Belo Horizonte (Lazzarini *et al.*, 2008) and 38% in Rio Grande (Von Groll *et al.*, 2010). Because this lineage was also detected in other parts of the world (Gibson *et al.*, 2008), it is not restricted to Brazil. Of the six clusters grouped by MIRU-VNTR analysis, four were formed by RD^{Rio} strains, and the other two clusters were composed of non-RD^{Rio} strains.

Similar to other studies, all RD^{Rio} lineage strains identified here belonged to the LAM family, which is responsible for 15% of TB cases worldwide (Brudey *et al.*, 2006; Lazzarini *et al.*, 2007; Lazzarini *et al.*, 2008; Von Groll *et al.*, 2010). We found that 81.25% of the LAM strains found in this study were from the RDRio sublineage, but more studies are needed to evaluate any possible selective advantages of LAM RD^{Rio} strains.

Conclusion

High genetic diversity within circulating *M. tuberculosis* was observed in this study and others; this feature may contribute to high TB prevalence (Silva *et al.*, 2009; Von Groll *et al.*, 2010). However, the absence of clonal dispersion may also indicate that effective medical treatment would result in reduced transmission. The RD^{Rio} sublineage has been present in Rio Grande-RS since 1998. The continued monitoring of RD^{Rio} in clinical isolates will elucidate its epidemiological significance.

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