Fitness study of the RD^{Rio} lineage and Latin American–Mediterranean family of *Mycobacterium tuberculosis* in the city of Rio Grande, Brazil

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Abstract

RD^{Rio} is a novel *Mycobacterium tuberculosis* lineage of the Latin American–Mediterranean (LAM) family. LAM has been found worldwide but is more predominant in South America. The aim of this study was to assess the presence of the RD^{Rio} lineage and LAM family in the city of Rio Grande, Brazil, and to investigate the fitness of these strains based on determination of their growth rate. Fifty clinical isolates of *M. tuberculosis* were genotyped and 43 different patterns were found by spoligotyping and mycobacterial interspersed repetitive units–variable number of tandem repeats. The predominant genotypes belonged to the LAM family (54% of the strains) followed by clade T (22%) and Haarlem (16%). The RD^{Rio} lineage represented 38% of the total strains and 70.4% of the LAM strains found in this study. Strains belonging to the LAM family showed a fitness advantage when comparing their rate of growth with that of non-LAM strains, but a significant difference between RD^{Rio} and non-RD^{Rio} strains was not confirmed.

Introduction

Tuberculosis remains one of the most important health problems and a leading cause of mortality worldwide. Ninety-nine per cent of the estimated 1.7 million deaths and 95% of the 9.2 million new cases in 2006 occurred in middle- and low-income countries, with Brazil ranking 15th among the 22 high-burden countries (World Health Organization, 2008). The city of Rio Grande, in the far south of Brazil, reported an incidence of tuberculosis of 75 in 100 000, which was 40% higher than in the rest of the country in 2006 (Health Ministry of Brazil, 2008). Assessing the molecular and phenotypic characteristics of the *Mycobacterium tuberculosis* strains present in this population could help to understand the factors related to the microorganism that could explain the higher incidence of the disease in this setting.

Molecular characterization has elucidated how *M. tuberculosis* has spread worldwide, and revealed the predominance of different genotypes in geographical regions and their possible adaptation to particular human populations (Brudey *et al.*, 2006; Filliol *et al.*, 2006; Gagneux *et al.*, 2006a). The evolution of *M. tuberculosis* has also been revealed through the study of irreversible genomic deletions and punctual mutations in strains with different phenotypic characteristics (Brosch *et al.*, 2002; Gutierrez *et al.*, 2005).

PCR-based typing methods such as spoligotyping and mycobacterial interspersed repetitive units–variable number of tandem repeats (MIRU-VNTR) have allowed the grouping of patterns according to their similarity and creating clades, genotypic families and/or lineages (Kamerbeek *et al.*, 1997; Supply *et al.*, 2001). The W-Beijing family is the most widely documented and it has been associated with high spread capacity, treatment failure, higher virulence and drug resistance (van Soolingen *et al.*, 1995; Bifani *et al.*, 1996; Toungoussova *et al.*, 2002; Lan *et al.*, 2003; López *et al.*, 2003; Hillemann *et al.*, 2005; Kubica *et al.*, 2005).

Recently, a novel *M. tuberculosis* lineage was identified as the major cause of tuberculosis in Rio de Janeiro, Brazil. The

RD^{Rio} lineage is a member of the Latin American–Mediterranean (LAM) family and presents a large deletion of 26 314 kb of contiguous DNA sequence with deletion or alteration of 10 genes, including two potential immunogenic proline-proline-glutamic acid (PPE) proteins (Lazzarini *et al.*, 2007). An association between RD^{Rio} lineage and a high prevalence of tuberculosis could be related to enhanced virulence and/or specific adaptation to the European–Latin American host population, as reported based on epidemiological and clinical findings (Lazzarini *et al.*, 2007); however, no study has assessed the fitness of this lineage compared with strains not belonging to RD^{Rio}. There are very few studies that have experimentally associated the fitness of a defined strain with its clinical phenotype (but see López *et al.*, 2003; Garcia de Viedma *et al.*, 2005; Theus *et al.*, 2005). Fitness studies may also help to understand the biology of the *M. tuberculosis* strain, which could have an influence on their dissemination and prevalence in certain settings.

The present study was undertaken to assess the presence of the RD^{Rio} lineage and LAM family in the city of Rio Grande, Brazil, and to investigate the fitness of these strains based on determination of their rate of growth.

Materials and methods

Samples

Fifty clinical isolates of *M. tuberculosis* obtained from patients of the National Control Program of TB in Rio Grande, Brazil, were studied (Honscha *et al.*, 2008). The majority of patients were male (72%) and were in the economically productive age group: 2% were aged 0–19 years, 18% 21–30 years, 30% 31–40 years, 27% 41–50 years, 16% 51–60 years and 7% > 60 years. With regard to the HIV status, 56% were negative, 4% were positive and 40% were HIV status unknown. The isolates were cultured at 37 °C on Ogawa–Kudoh medium (Kudoh & Kudoh, 1974) and stored at − 70 °C in 25% glycerol in water. Drug susceptibility testing (DST) data to first-line drugs were available from Sanchotene *et al.* (2008).

Extraction of genomic DNA

One loopful of culture was transferred to a microtube containing 300 μL of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The bacterial suspension was inactivated at 80 °C for 30 min and centrifuged at 5000 *g* for 5 min. The supernatant was collected, aliquoted in 50-μL volumes and stored at − 20 °C.

Determination of gene mutations associated with drug resistance

DST results based on phenotypic methods revealed only one strain (06-235) to be multidrug resistant (MDR). DNA

sequencing was performed to look for mutations in the genes *rpoB*, *katG* and the *inhA* promoter associated with resistance to rifampicin and isoniazid. For rifampicin, primers *rpoB*geneSAnew and *rpoB*geneRA were used as described (Rigouts *et al.*, 2007). For isoniazid, three sets of primers were used, TB84/TB85, TB86/TB87 and TB88/TB89, to amplify three different regions of the *katG* gene and TB92/TB93 primers for the *inhA* promoter (Telenti *et al.*, 1997; Kiepiela *et al.*, 2000). The primers and their position in *katG* are detailed in Table 1.

Genotyping by spoligotyping

Spoligotyping was performed with a commercial kit (Ocimum Biosolutions BV, India) according to standard procedures (Kamerbeek *et al.*, 1997). The patterns observed were recorded in binary format (1 = presence and 0 = absence of the spacer) for analysis of genetic relationships. The

Table 1. Primers used in the study

Primer	Target	Position in *katG*	Fragment size (bp)
TB84: 5′-CCGGCACCTAC CGCATCCAC-3′	*katG*	329–348	269
TB85: 5′-GCCCCAATAGA CCTCATCGG-3′		577–597	
TB86: 5′-GAAACAGCGGCGGCG CTGGATCGT-3′	*katG*	781–804	209
TB87: 5′-GTTGTCCCATTA CGTCGGGG-3′		971–990	
TB88: 5′-CGACGATGCTGGC CACTGAC-3′	*katG*	1124–1143	611
TB89: 5′-TTGTTCCTGC GACGCATCGTG-3′		1714–1734	
TB92: 5′-CCTCGCTGC CCAGAAAGGGA-3′	*inhA* promoter	–	248
TB93: 5′-ATCCCCC GGTTTCCTCCGGT-3′		–	
*rpoB*geneSAnew: 5′-GCAAAACAGCCGCT AGTCCTAGTCCGA-3′	*rpoB*	–	2106
*rpoB*geneRA: 5′-GCGCCATCTCGCC GTCGTCAGTACAG-3′		–	
IS1561F: 5′-GACCTGACGCCGCTGACAC-3′	IS*1561′* (*Rv3349c*)	–	530
IS1561R: 5′-CACCTACACCGCTTCCTGCC-3′		–	
RD^{Rio}BrgF: 5′-CACTCCGGCTGCCAATCTCGTC-3′	RD^{Rio} Bridge	–	1175
RD^{Rio}BrgR: 5′-CACCGCCACGCTGAATGAGACCA-3′		–	

genotypes were classified according to SpolDB4 (Brudey *et al.*, 2006) and compared against the SITVIT database (http://www.pasteur-guadeloupe.fr:8081/SITVITDemo/) to identify shared types (ST).

Genotyping by MIRU-VNTR

MIRU-VNTR was carried out using the 12-loci format as previously described (Supply *et al.*, 2000). The results from each locus (2, 4, 10, 16, 20, 23, 24, 26, 27, 37, 31 and 40) were combined to form a 12-digit allele profile for analysis of genetic relationships.

Analysis of genetic relationships

Construction of dendrograms was performed via http://www.miru-vntrplus.org (Allix-Béguec *et al.*, 2008) using clustering with the Unweighted Pair Group Method with Arithmetic Mean. Clusters were defined as at least two *M. tuberculosis* strains with identical patterns isolated from different patients. The discriminatory power of spoligotyping, MIRU-VNTR and the two in combination was calculated using the Hunter–Gaston discriminatory index (HGDI) (Hunter & Gaston, 1988).

Identification of strains belonging to the RD^{Rio} lineage

A multiplex PCR adapted from Gibson *et al.* (2008) was performed to differentiate strains belonging to the RD^{Rio} lineage. The PCR reaction was performed in a final volume of 30 μL, containing $1 \times$ buffer supplemented with 1.5 mM MgCl$_2$, $1 \times$ solution Q and 0.6 U HotStarTaq DNA Polymerase (Qiagen), 400 nM of each of primers RD^{Rio}Brg and IS*1561* (both forward and reverse; Table 1), 200 mM dNTPs and 1.5 μL DNA suspension. The cycle conditions were 95 °C for 10 min, followed by 35 cycles at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 4 min, and a final extension at 72 °C for 10 min. PCR products were detected by 1.6% agarose gel electrophoresis, followed by UV detection using ethidium bromide. Identification of the RD^{Rio} lineage was established according to band size. A band size of 1175 bp corresponded to the RD^{Rio} lineage and a band of 530-bp corresponded to non-RD^{Rio}.

Fitness studies

Of the 50 strains molecularly characterized, 40 were available to perform the fitness study. Fresh subcultures were prepared on Ogawa–Kudoh medium and kept for 3 weeks. An inoculum was prepared at a turbidity of a McFarland tube No. 1, weighting 1 mg of bacteria culture per 1 mL of ultrapure water. In order to prepare a homogeneous inoculum, a loopful of culture was transferred to a vial containing glass beads and 500 μL of ultrapure water was added. The vial was shaken in a vortex mixer for 40 s; additional water was

then added to complete make 1 mg mL^{-1} and shaken again for 30 s. The inoculum was diluted 1 : 20 in Middlebrook 7H9 broth supplemented with 0.1% casitone, 0.5% glycerol and 10% OADC (oleic acid, albumin, dextrose and catalase) (Becton-Dickinson). Cultures were started in 96-well flat-bottom plates containing 100 μL of 7H9 broth + 100 μL of the inoculum in triplicate wells. Two hundred microlitres of 7H9 broth alone was added to separate triplicate wells as negative control. The plate was closed with its lid and incubated in a sealed plastic bag at 37 °C. After 48 h, 30 μL of 0.02% resazurin (Acros Organic NV, Belgium) was added to all test wells and reincubated at 37 °C. Reduction of resazurin, which is dependent on bacterial metabolic activity, and turns the indicator from blue to pink (resorufin), was assessed every 24 h by measuring the reduction in the OD$_{620 nm}$ in each well using a plate reader (TECAN Spectrum Classic). Growth curves were obtained by plotting the difference in OD between test and control wells vs. the time of incubation. To assess the rate of growth as reported by Toungoussova *et al.* (2004), we also used the time in hours for doubling of the OD value from 0.2 to 0.4. This value was calculated from the growth curve considering that all strains were in the logarithmic phase of growth between these two values. Using this approach it was possible to compare the fitness of each strain based on their respective rate of growth.

Statistical analysis

Differences in rate of growth between strains of the RD^{Rio} and non-RD^{Rio} lineage and between strains belonging to the LAM and non-LAM family were calculated with an independent samples *t*-test with two-tailed probability. Difference were considered significant at $P < 0.05$. Analysis was performed with MEDCAL$^®$ software (v9.6.2.0; Mariakerke, Belgium).

Results

Sequencing for gene mutations associated with drug resistance

Only one MDR strain was found. A mutation was identified at Ser531Trp of the *rpoB* gene associated with resistance to rifampicin. For isoniazid, the *katG* gene and *inhA* promoter were investigated. No mutation was detected in the *inhA* promoter. For *katG*, the PCR reaction was negative when amplifying the hotspot region for mutations conferring resistance to isoniazid (781–990 bp). Two additional regions of the *katG* gene were assessed (329–597 and 1124–1734 bp) and the PCR reactions were again negative.

Spoligotyping

Twenty-seven different patterns were obtained by spoligotyping. Seventeen strains had unique patterns and 33 strains were

grouped in 10 different clusters of two to six strains. The HGDI was 0.960. According to the classification based in SpolDB4 (Brudey *et al.*, 2006), the predominant genotypes were the LAM family (54% of the strains) followed by clade T (22%) and Haarlem (16%); three strains had unknown profiles and one strain was characterized as of the East African–Indian (EAI) family. The strains were also compared with the SITVIT database: the predominant ST were ST17 (12%), ST42 (10%), ST45 (10%) and ST53 (8%). Four patterns, one of them shared by two strains, were not found in the database. At the time of the analysis, the database contained 39 609 entries from 121 countries of isolation and 1939 different STs.

MIRU-VNTR assay

Thirty-nine different patterns were detected by MIRU-VNTR, with 15 strains distributed in nine different clusters of two or three strains and 30 strains had unique patterns. The HGDI was 0.989.

Spoligotyping and MIRU-VNTR combined

When the two methods were analysed in combination, the HGDI was 0.993 with 43 different patterns being discriminated (Fig. 1). Thirty-seven strains had unique patterns and 13 strains were grouped in six different clusters of two or three strains.

Identification of RD^{Rio} strains

Of the 50 strains analysed, 19 (38%) were identified as belonging to the RD^{Rio} lineage, including the MDR strain 06-235, and the other 31 strains were grouped as non-RD^{Rio}. All 19 RD^{Rio} lineage strains belonged to the LAM family based on spoligotyping, representing 70.4% of the total number of LAM strains found in this study. The LAM subfamilies found in the RD^{Rio} lineage were LAM 2 (42.1%), LAM 9 (31.6%), LAM 1 (15.8%), LAM 4 (5.3%) and LAM 5 (5.3%). Of the six clusters grouped by spoligotyping and MIRU-VNTR, two were formed by RD^{Rio} strains and the other four clusters for non-RD^{Rio}.
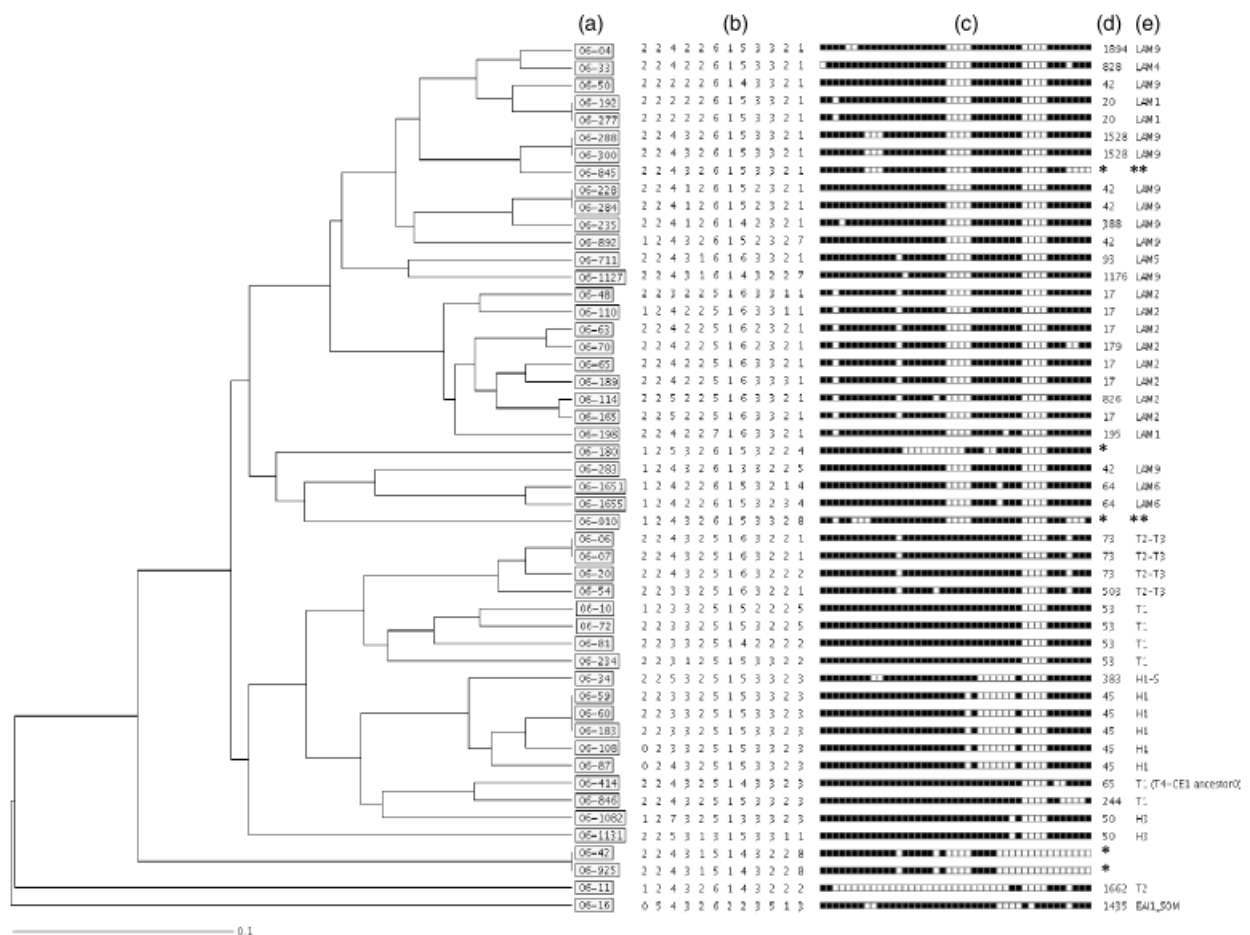

Fig. 1. (a) Dendrogram with genotypic patterns of MIRU-VNTR and spoligotyping provided by the MIRU-VNTRplus site; (b) sequence of allele number found in the 12 loci MIRU-VNTR; (c) spoligotype patterns; (d) number of STs of the SITVIT database; (e) subfamily based in the SpolDB4. *Patterns not found in the SITVIT; **subfamilies not identified in the SpolDB4 but classified as LAM 9 based on pattern.

Fitness study comparing the rate of growth of RD^{Rio} and non-RD^{Rio} strains

Forty strains were available for assessing fitness. Strains 06-198 and 06-228 (RD^{Rio}) and 06-016, 06-072, 06-234, 06-300, 06-892, 06-925, 06-1651 and 06-1655 (non-RD^{Rio}) were not included due to difficulty in subcultivation. Figure 2 shows the growth curve of six RD^{Rio} strains including the MDR strain 06-235. The rate of growth of each strain is given in Table 2. The average rate of growth of RD^{Rio} strains was 32.2 h compared with 35.8 h for non-RD^{Rio} strains; however, this difference was not statistically significant (Table 3). Within this group, the MDR strain had the slowest rate of growth in the RD^{Rio} lineage.

Fitness study comparing the rate of growth of LAM and non-LAM strains

The rate of growth was also compared between strains of the LAM family ($n = 21$) with those of non-LAM ($n = 19$) strains. The LAM group consisted of the same 17 clinical RD^{Rio} isolates plus isolates 06-283, 06-288, 06-910 and 06-1127. The average rate of growth of the LAM strains was 30.8 h compared with 38.1 h for the non-LAM strains, and the MDR strain had the slowest rate of growth among the LAM strains. LAM strains had a significantly faster rate of growth compared with non-LAM strains (Table 3).

Discussion

Two genotyping methods were applied here: spoligotyping and 12-loci MIRU-VNTR. The HGDI by spoligotyping, MIRU-VNTR and the two methods combined was 0.960, 0.989 and 0.993, respectively. These results confirm the recommendation of using the two methods combined for obtaining high discriminatory power (Sola *et al.*, 2003). Two factors may influence this high discriminatory power: high
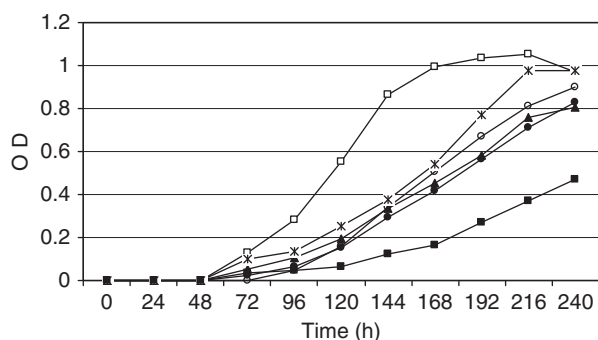

Fig. 2. Growth curves of six *Mycobacterium tuberculosis* strains measured based on reduction of resazurin [●, 06-004; □, 06-033; ▲, 06-048; ○, 06-050; ✳, 06-063; ■, 06-235 (MDR)]. The rate of growth was assessed by the time needed by each strain to reach an OD of 0.4 starting at an OD of 0.2.

Table 2. Classification of the lineage, genotype and rate of growth (OD doubling time) of the strains studied

Strain	Lineage	Genotype family by spoligotype	OD doubling time (h)
06-04	RD^{Rio}	LAM 9	36.4
06-33	RD^{Rio}	LAM 4	23.4
06-48	RD^{Rio}	LAM 2	36.4
06-50	RD^{Rio}	LAM 9	27.0
06-63	RD^{Rio}	LAM 2	38.7
06-65	RD^{Rio}	LAM 2	29.8
06-70	RD^{Rio}	LAM 2	22.9
06-110	RD^{Rio}	LAM 2	40.3
06-114	RD^{Rio}	LAM 2	24.5
06-165	RD^{Rio}	LAM 2	39.4
06-189	RD^{Rio}	LAM 2	21.1
06-192	RD^{Rio}	LAM 1	17.1
06-198	RD^{Rio}	LAM 1	ND
06-228	RD^{Rio}	LAM 9	ND
06-235	RD^{Rio}	LAM 9	47.6
06-277	RD^{Rio}	LAM 1	38.1
06-284	RD^{Rio}	LAM 9	27.0
06-711	RD^{Rio}	LAM 5	36.3
06-845	RD^{Rio}	LAM 9	41.7
06-06	Non-RD^{Rio}	Clade T2/T3	33.8
06-07	Non-RD^{Rio}	Clade T2/T3	25.6
06-10	Non-RD^{Rio}	Clade T1	27.9
06-11	Non-RD^{Rio}	Unknown	39.1
06-16	Non-RD^{Rio}	EAI	ND
06-20	Non-RD^{Rio}	Clade T2/T3	44.1
06-34	Non-RD^{Rio}	Haarlem 1-S	36.9
06-42	Non-RD^{Rio}	Unknown	42.9
06-54	Non-RD^{Rio}	Clade T2/T3	30.5
06-59	Non-RD^{Rio}	Haarlem 1	42.5
06-60	Non-RD^{Rio}	Haarlem 1	17.3
06-72	Non-RD^{Rio}	Clade T1	ND
06-81	Non-RD^{Rio}	Clade T1	34.4
06-87	Non-RD^{Rio}	Haarlem 1	36.9
06-108	Non-RD^{Rio}	Haarlem 1	45.3
06-180	Non-RD^{Rio}	Unknown	39.5
06-183	Non-RD^{Rio}	Haarlem 1	42.8
06-234	Non-RD^{Rio}	Clade T1	ND
06-283	Non-RD^{Rio}	LAM 9	31.1
06-288	Non-RD^{Rio}	LAM 9	21.8
06-300	Non-RD^{Rio}	LAM 9	ND
06-414	Non-RD^{Rio}	Clade T1	42.6
06-846	Non-RD^{Rio}	Clade T1	53.8
06-892	Non-RD^{Rio}	LAM 9	ND
06-910	Non-RD^{Rio}	LAM	21.7
06-925	Non-RD^{Rio}	Unknown	ND
06-1082	Non-RD^{Rio}	Haarlem 3	30.0
06-1127	Non-RD^{Rio}	LAM 9	25.0
06-1131	Non-RD^{Rio}	Haarlem 3	57.5
06-1651	Non-RD^{Rio}	LAM 6	ND
06-1655	Non-RD^{Rio}	LAM 6	ND

ND, not determined.

clonal diversity present in the population and the low cluster number, reflecting few cases of recent transmission. However, it is important to consider that the small number of

Table 3. Comparison of the rate of growth between RD^{Rio} and non-RD^{Rio} lineage members (Analysis 1) and between strains belonging to LAM and non-LAM family (Analysis 2)

	Lineage	No. of samples	Median (h)	95% CI	*P*
Analysis 1	RD^{Rio}	17	32.2	27.7–36.8	0.2505
	Non-RD^{Rio}	23	35.8	31.4–40.2	
Analysis 2	LAM	21	30.8	27.0–34.7	0.0153*
	Non-LAM	19	38.1	33.5–42.7	

*Significant difference of the rate of growth ($P < 0.05$) by *t*-test.

samples may underestimate the number of clusters and the role of recent transmission (Glynn *et al.*, 1999).

The patterns obtained by spoligotyping were classified based on the profiles present in the SpolDB4. The predominant genotypes were of the LAM family ($n = 27$ strains), clade T ($n = 11$) and Haarlem family ($n = 8$). Three isolates presented an 'unknown' profile and one was classified as belonging to the EAI family. The predominance of LAM, clade T and Haarlem in South America has been reported previously (Brudey *et al.*, 2006; Lazzarini *et al.*, 2008). The interesting finding is the presence of one EAI strain in the population of Rio Grande. This family is referred to as 'ancestral' due to the fact that they conserve the TbD1 genomic region (Brosch *et al.*, 2002), and it is highly prevalent in Asia and Oceania but considered exotic in South America. The presence of this strain in Rio Grande might be explained by the fact that it is an important sea port, which receives ships from different parts of the world, including Asia.

Recently, a new lineage was reported associated with a high incidence of tuberculosis in Rio de Janeiro (Lazzarini *et al.*, 2007). This lineage, named RD^{Rio}, has also been identified in other parts of the world (Gibson *et al.*, 2008). In the present study, of the 50 strains evaluated, 19 (38%) were identified as belonging to the RD^{Rio} lineage. All RD^{Rio} strains belonged to the LAM family, with LAM 2 being the most commonly found. LAM 9 was found in both lineages almost in the same proportion: 55% were RD^{Rio} and 45% were non-RD^{Rio}. In previous studies the presence of the RD^{Rio} lineage in Brazil was 30% in Rio de Janeiro (Lazzarini *et al.*, 2007) and 38% in Belo Horizonte (Lazzarini *et al.*, 2008). Seventy per cent of the LAM 9 strains in Rio de Janeiro belonged to the RD^{Rio} lineage and more than 90% in Belo Horizonte. Lazzarini *et al.* (2008) reported that the possible origin of RD^{Rio} is from a single ancestor with an LAM 9 spoligotype signature, and given that they found a higher proportion of LAM 9 RD^{Rio} compared with LAM 9 non-RD^{Rio}, they propose that the RD^{Rio} lineage could be more transmissible.

An assessment of the fitness of these strains was performed to see if there was any difference in fitness between RD^{Rio} and non-RD^{Rio} strains and also between LAM and

non-LAM strains. There is no standard method to compare fitness in the laboratory. A growth curve has been considered as an optimal marker to compare fitness in other bacteria (Laurent *et al.*, 2001) but in *M. tuberculosis* it is considered a difficult method due to its slow growth and tendency to form clumps when grown in liquid media (Lambrecht *et al.*, 1988). In the present study we set up a new method to determine the growth curve of *M. tuberculosis* by measuring quantitatively the reduction of resazurin based on OD. Resazurin is a redox indicator that is reduced to resorufin by metabolically active cells (Ali-Vehmas *et al.*, 1991). In *M. tuberculosis* resazurin has been used successfully to test the susceptibility of active and dormant bacilli to antituberculosis drugs by the resazurin microtitre assay plate (Palomino *et al.*, 2002; Martin *et al.*, 2003; Taneja & Tyagi, 2007). There is a direct correlation between the reduction of resazurin in the growth medium and the extent of proliferation of live organisms (O'Brien *et al.*, 2000).

Using this method it was possible to compare the rate of growth of 40 strains; the rate of growth was the parameter selected for comparing fitness based on the study of Toungoussova *et al.* (2004). The growth curve was assessed by a metabolic method, the BACTEC MGIT 960 system, and the rate of growth was expressed in hours. The present data were analysed comparing the RD^{Rio} and non-RD^{Rio} lineage and also comparing strains belonging to the LAM and non-LAM family. In both cases, the MDR strain presented the slowest rate of growth among the strains evaluated. For both RD^{Rio} and LAM genotypes the MDR strain had the slowest rate of growth, increasing the average value for the group. It is often assumed that organisms pay a physiological cost for acquiring drug resistance. The MDR strain 06-235 presented the Ser531Trp mutation in *rpoB*. In clinical isolates from Brazil, this mutation has a frequency of 4% (Valim *et al.*, 2000). However, the Ser531Leu (TCG to TTG) mutation is the most frequent, being present in 50% of the mutants; it is also the most frequently found mutation *in vitro* (Billington *et al.*, 1999; Morlock *et al.*, 2000). Fitness studies have shown a biological cost of *M. tuberculosis* resistance to rifampicin based on *in vitro* selected mutations compared with the identical genetic background strain. However, the smallest deficit was seen in strains with the mutation Ser531Leu (Billington *et al.*, 1999; Gagneux *et al.*, 2006b). Clinical strains with this mutation presented equal or greater fitness in comparison with susceptible strains (Toungoussova *et al.*, 2004; Gagneux *et al.*, 2006b). This does not occur with the mutation Ser531Trp, which showed a high fitness cost in both clinical and *in vitro* mutants (Mariam *et al.*, 2004; Gagneux *et al.*, 2006b). In addition to the mutation in *rpoB*, an additional factor in the fitness disadvantage of the 06-235 strain could be a deletion in the *katG* gene. The *katG* gene encodes a catalase-peroxidase enzyme that transforms the prodrug isoniazid into its active form. MDR strains generally

show mutations in *katG* but there is the rare possibility of gene deletion (Zhang *et al.*, 1992) where the strain loses the catalase-peroxidase activity. The deletion of *katG* has a marked biological cost because this enzyme protects the bacteria against the oxidative stress produced by macrophage metabolism (Pym *et al.*, 2002). Another important cause of resistance to isoniazid is a mutation in *inhA* or its promoter region, with promoter mutations being more frequent than mutations in the structural gene (Musser *et al.*, 1996). In the present study, the MDR strain did not present a mutation in the promoter of *inhA*. Mutations in the *inhA* promoter and structural gene are associated more with isoniazid mono-resistance then MDR (Hazbón *et al.*, 2006).

We verified a fitness advantage by comparing the rate of growth of strains belonging to the LAM family with other families found in the population. The rate of growth is associated with the capacity for reproduction of the bacteria. Strains that are metabolically more active reproduce more rapidly. In infectious processes, bacterial load is an important factor in infection and its transmission. The LAM family is found worldwide but it is more predominant in South America, corresponding to about 50% of the tuberculosis burden in this continent (Brudey *et al.*, 2006). The presence of RD^Rio strains as an LAM subgroup has been suggested as a factor that increases the spread of the LAM family. In the present study, despite the RD^Rio strains comprising 70.4% of the total LAM family found in the city of Rio Grande, we could not find a statistically significant difference in the rate of growth between RD^Rio and non-RD^Rio strains. A bias due to the small number of samples could be present in this study, but the equilibrium between RD^Rio and non-RD^Rio in the LAM 9 subfamily as well as in the number of clusters found in this population are in line with the fitness results; however, these results reflect what occurs *in vitro*, and a different situation could certainly occur in the host.

In conclusion, there is epidemiologic evidence that certain genotypes may be more transmissible in different geographical regions due to an adaptation for determined host ethnicity (Gagneux & Small, 2007; Hanekom *et al.*, 2007). We confirmed *in vitro* a fitness advantage of the predominant genotypic family in the city of Rio Grande. Strains belonging to the LAM family showed a faster growth compared with other families. RD^Rio strains were predominant in the LAM family, but a difference in fitness between RD^Rio and non-RD^Rio strains was not confirmed. Additional studies must be performed to confirm if the predominance of the LAM family in certain population is due to a fitness advantage of LAM strains or RD^Rio strains.

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References

Ali-Vehmas T, Louhi M & Sandholm M (1991) Automation of the resazurin reduction test using fluorometry of microtitration trays. *J Vet Med* **38**: 358–372.

Allix-Béguec C, Harmsen D, Weniger T, Supply P & Niemann S (2008) Evaluation and user-strategy of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of *Mycobacterium tuberculosis* complex isolates. *J Clin Microbiol* **46**: 2692–2699.

Bifani PJ, Plikaytis BB, Kapur V *et al.* (1996) Origin and interstate spread of a New York City multidrug-resistant *Mycobacterium tuberculosis* clone family. *JAMA* **275**: 452–457.

Billington OJ, McHugh TD & Gillespie SH (1999) Physiological cost of rifampin resistance induced *in vitro* in *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* **43**: 1866–1869.

Brosch R, Gordon SV, Marmiesse M *et al.* (2002) A new evolutionary scenario for the *Mycobacterium tuberculosis* complex. *P Natl Acad Sci USA* **99**: 3684–3689.

Brudey K, Driscoll JR, Rigouts L *et al.* (2006) *Mycobacterium tuberculosis* complex genetic diversity: mining the fourth international spoligotyping database (SpolDB4) for classification, population genetics and epidemiology. *BMC Microbiol* **6**: 23.

Filliol I, Motiwala AS, Cavatore M *et al.* (2006) Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *J Bacteriol* **188**: 759–772.

Gagneux S & Small P (2007) Global phylogeography of *Mycobacterium tuberculosis* and implications for tuberculosis product development. *Lancet Infect Dis* **7**: 328–337.

Gagneux S, DeRiemer K, Van T *et al.* (2006a) Variable host–pathogen compatibility in *Mycobacterium tuberculosis*. *P Natl Acad Sci USA* **103**: 2869–2873.

Gagneux S, Long CD, Small PM, Van T, Schoolnik GK & Bohannan BJM (2006b) The competitive cost of antibiotic resistance in *Mycobacterium tuberculosis*. *Science* **312**: 1944–1946.

Garcia de Viedma D, Lorenzo G, Cardona PJ, Rodriguez NA, Gordillo S, Serrano MJR & Bouza E (2005) Association between the infectivity of *Mycobacterium tuberculosis* strains and their efficiency for extrarespiratory infection. *J Infect Dis* **192**: 2059–2065.

Gibson AL, Huard RC, van Pittius NCG *et al.* (2008) Application of sensitive and specific molecular methods to uncover global dissemination of the major RD^Rio sublineage of the Latin American–Mediterranean *Mycobacterium tuberculosis* spoligotype family. *J Clin Microbiol* **46**: 1259–1267.

Glynn JR, Vynnycky E & Fine PE (1999) Influence of sampling on estimates of clustering and recent transmission of *Mycobacterium tuberculosis* derived from DNA fingerprinting techniques. *Am J Epidemiol* **149**: 366–371.

Gutierrez MC, Brisse S, Brosch R, Fabre M, Omaïs B, Marmiesse M, Supply P & Vincent V (2005) Ancient origin and gene mosaicism of the progenitor of *Mycobacterium tuberculosis*. *PLoS Pathog* **1**: e5 DOI: 10.1371/journal.ppat.0010005.

Hanekom M, van der Spuy GD, van Pittius NCG, McEvoy CRE, Ndabambi SL, Victor TC, Hoal EG, van Helden PD & Warren RM (2007) Evidence that the spread of *Mycobacterium tuberculosis* strains with the Beijing genotype is human population dependent. *J Clin Microbiol* **45**: 2263–2266.

Hazbón MH, Brimacombe M, del Valle MB *et al.* (2006) Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrob Agents Ch* **50**: 2640–2649.

Health Ministry of Brazil (2008) Sistema de informação de agravos de notificação. Available at http://dtr2004.saude.gov. br/sinanweb/novo. Accessed on 3 October 2008.

Hillemann D, Kubica T, Rüsch-Gerdes S & Niemann S (2005) Disequilibrium in distribution of resistance mutations among *Mycobacterium tuberculosis* Beijing and non-Beijing strains isolated from patients in Germany. *Antimicrob Agents Chemother* **49**: 1229–1231.

Honscha G, Von Groll A, Valença M, Ramos DF, Sanchotene K, Scaini CJ, Ribeiro MO & Silva PEA (2008) The laboratory as a tool to qualify tuberculosis diagnosis. *Int J Tuberc Lung D* **12**: 218–220.

Hunter PR & Gaston MA (1988) Numerical índex of discriminatory ability of typing system: an application of Simpson's índex of diversity. *J Clin Microbiol* **26**: 2465–2466.

Kamerbeek J, Schouls L, Kolk A *et al.* (1997) Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* **35**: 907–914.

Kiepiela P, Bishop KS, Smith AN, Roux L & York DF (2000) Genomic mutations in the katG, inhA and ahpC genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. *Int J Tuberc Lung D* **80**: 47–56.

Kubica T, Agzamova R, Wright A, Aziz MA, Rakishev G, Bismilda V, Richter E, Rüsch-Gerdes S & Niemann S (2005) The Beijing genotype is a major cause of drug-resistant tuberculosis in Kazakhstan. *Int J Tuberc Lung D* **9**: 646–653.

Kudoh S & Kudoh T (1974) A simple technique for culturing tubercle bacilli. *B World Health Organ* **51**: 71–82.

Lambrecht RS, Carriere JF & Collins MT (1988) A model for analysing growth kinetics of a slowly growing *Mycobacterium sp. Appl Environ Microb* **54**: 910–916.

Lan NT, Lien HT, Tung LB, Borgdorff MW, Kremer K & van Soolingen D (2003) *Mycobacterium tuberculosis* Beijing genotype and risk for treatment failure and relapse, Vietnam. *Emerg Infect Dis* **9**: 1633–1635.

Laurent F, Lelièvre H, Cornu M, Vandenesch F, Carret G, Etienne J & Flandrois J (2001) Fitness and competitive growth advantage of new gentamicin-susceptible MRSA clones spreading in French hospitals. *J Antimicrob Chemoth* **47**: 277–283.

Lazzarini LCO, Huard RC, Boechat NL *et al.* (2007) Discovery of a novel *Mycobacterium tuberculosis* lineage that is a major cause of tuberculosis in Rio de Janeiro, Brazil. *J Clin Microbiol* **45**: 3891–3902.

Lazzarini LCO, Spindola SM, Bang H, Gibson AL, Weisenberg S, Carvalho WS, Augusto CJ, Huard RC, Kritski AL & Ho JL (2008) RDRio *Mycobacterium tuberculosis* infection is associated with a higher frequency of cavitary pulmonary disease. *J Clin Microbiol* **46**: 2175–2183.

López B, Aguilar D, Orozco H *et al.* (2003) A marked difference in pathogenesis and immune response induced by different *Mycobacterium tuberculosis* genotypes. *Clin Exp Immunol* **133**: 30–37.

Mariam DH, Mengistu Y, Hoffner SE & Andersson DI (2004) Effect of rpoB mutations conferring rifampin resistance on fitness of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **48**: 1289–1294.

Martin A, Camacho M, Portaels F & Palomino JC (2003) Resazurin microtiter assay plate testing of *Mycobacterium tuberculosis* susceptibilities to second-line drugs: rapid, simple and inexpensive method. *Antimicrob Agents Chemother* **47**: 3616–3619.

Morlock GP, Plikaytis BB & Crawford JT (2000) Characterization of spontaneous, *in vitro*-selected, rifampin-resistant mutants of *Mycobacterium tuberculosis* strain H37Rv. *Antimicrob Agents Chemother* **44**: 3298–3301.

Musser JM, Kapur V, Williams DL, Kreiswirth BN, van Soolingen D & van Embden JD (1996) Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. *J Infect Dis* **173**: 196–202.

O'Brien J, Wilson I, Orton T & Pognan F (2000) Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* **267**: 5421–5426.

Palomino JC, Martin A, Camacho M, Guerra H, Swings J & Portaels F (2002) Resazurin microtiter assay plate: simple and inexpensive method for detection of drug resistance *Micobacterium tuberculosis*. *Antimicrob Agents Chemother* **46**: 2720–2722.

Pym AS, Saint-Joanis B & Cole ST (2002) Effect of *katG* mutations on the virulence of *Mycobacterium tuberculosis* and the implication for transmission in humans. *Infect Immun* **70**: 4955–4960.

Rigouts L, Nolasco O, Rijk P, Nduwamahoro E, Van Deun A, Ramsay A, Arevalo J & Portaels F (2007) Newly developed primers for comprehensive amplification of the rpoB gene and detection of rifampin resistance in *Mycobacterium tuberculosis*. *J Clin Microbiol* **45**: 252–254.

Sanchotene KO, Von Groll A, Ramos DF, Scholante AB, Honscha G, Valença M, Scaini CJ & Silva PEA (2008) Comparative evaluation of the nitrate reductase assay and the resazurin microtitre assay for drug susceptibility testing of *Mycobacterium tuberculosis* against first line anti-tuberculosis drugs. *Braz J Microbiol* **39**: 16–20.

Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P & Rastogi N (2003) Genotyping of the *Mycobacterium tuberculosis* complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. *Infect Genet Evol* **3**: 125–133.

Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B & Locht C (2000) Variable human minisatellite-like regions in the *Mycobacterium tuberculosis* genome. *Mol Microbiol* **36**: 762–771.

Supply P, Lesjean S, Savine E, Kremer K, van Soolingen D & Locht C (2001) Automated high-throughput genotyping for study of global epidemiology of *Mycobacterium tuberculosis* based on mycobacterial interspersed repetitive units. *J Clin Microbiol* **39**: 3563–3571.

Taneja NK & Tyagi JS (2007) Resazurin reduction assays for screening of anti-tubercular compounds against dormant and actively growing *Mycobacterium tuberculosis*, *Mycobacterium bovis* BCG and *Mycobacterium smegmatis*. *J Antimicrob Chemoth* **60**: 288–293.

Telenti A, Honoré N, Bernasconi C, March J, Ortega A, Heym B, Takiff HE & Cole ST (1997) Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J Clin Microbiol* **35**: 719–723.

Theus SA, Cave MD & Eisenach KD (2005) Intracellular macrophage growth rates and cytokine profiles of *Mycobacterium tuberculosis* strains with different transmission dynamics. *J Infect Dis* **191**: 453–460.

Toungoussova OS, Sandven P, Mariandyshev AO, Nizovtseva NI, Bjune G & Caugant DA (2002) Spread of drug-resistant *Mycobacterium tuberculosis* strains of the Beijing genotype in the Archangel Oblast, Russia. *J Clin Microbiol* **40**: 1930–1937.

Toungoussova OS, Caugant DA, Sandven P, Mariandyshev AO & Bjune G (2004) Impact of drug resistance on fitness of *Mycobacterium tuberculosis* strains of the W-Beijing genotype. *FEMS Immunol Med Mic* **42**: 281–290.

Valim AR, Rossetti ML, Ribeiro MO & Zaha A (2000) Mutations in the *rpoB* gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from Brazil. *J Clin Microbiol* **38**: 3119–3122.

van Soolingen D, Qian L, Haas PE, Douglas JT, Traore H, Portaels F, Qing HZ, Enkhsaikan D, Nymadawa P & van Embden JD (1995) Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. *J Clin Microbiol* **33**: 3234–3238.

World Health Organization (2008) *Global Tuberculosis Control – Surveillance, Planning, Financing*. World Health Organization, Geneva, Switzerland. Available at http://www.who.int.

Zhang Y, Heym B, Allen B, Young D & Cole S (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358**: 591–593.