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Mycobacterium tuberculosis DNA fingerprint clusters and its relationship with RD^{Rio} genotype in Brazil

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SUMMARY

Mycobacterium tuberculosis (Mtb) strains designated as RD^{Rio} are responsible for a large cluster of new cases of tuberculosis (TB) in Rio de Janeiro. They were previously shown to be associated with severe manifestations of TB. Here, we used three genotyping methods (IS*6110* RFLP, spoligotyping, and multiplex PCR) to characterize RD^{Rio} and non-RD^{Rio} strains from the metropolitan area of Vitória, State of Espirito Santo in southeast Brazil to determine strain diversity and transmission patterns. Strains with identical IS*6110* RFLP patterns were considered to belong to a cluster indicative of recent transmission. Between 2000 and 2010, we identified 5470 new TB patients and genotyped 981 Mtb strains. Of these, 376 (38%) were RD^{Rio}. By RFLP, 180 (48%) of 376 RD^{Rio} strains and 235 (40%) of 593 non-RD^{Rio} strains belonged to RFLP cluster pattern groups ($p = 0.023$). Simpson's diversity index based on RFLP patterns was 0.96 for RD^{Rio} and 0.98 for non-RD^{Rio} strains. Thus, although RD^{Rio} strains appear to be comprised of a fewer number of RFLP genotypes, they represent a heterogeneous group. While TB cases caused by RD^{Rio} appear more likely to be due to recent transmission than cases caused by non-RD^{Rio} strains, the difference is small. These observations suggest that factors other than inherent biological characteristic of RD^{Rio} lineages are more important in determining recent transmission, and that public health measures to interrupt new transmissions need to be emphasized for TB control in Vitória.

Keywords

Tuberculosis; DNA fingerprinting; *Mycobacterium tuberculosis*; Epidemiologic surveillance; RD^{Rio}

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1. Introduction

In 2004, the Brazilian National Tuberculosis Control Program designated 315 cities as priority regions for surveillance, control and prevention of TB,[1,2] including eight municipalities in the State of Espírito Santo, in southeastern Brazil. Despite efforts to improve the control program such as the introduction of directly observed therapy-short course (DOTS) and the family health program, the TB incidence has remained almost unchanged in Espírito Santo State over the past 10 years.[1,3] One possible explanation could be that active case finding efforts to interrupt new transmissions in high-risk populations have been inadequate. Another possible explanation may be that there were undetected outbreaks or spread of some highly transmissible strains in the state.[4]

In 2000, in collaboration with the State Health Department, the University of Espírito Santo implemented a laboratory network to improve TB diagnosis through standardized training of laboratory personnel and utilization of culture for all suspected TB diagnoses. As part of this strategy an information system entitled “TB-Notes” was created based on Lotus Notes™. This network includes four municipalities that report 70% of TB cases in the state, including Vitória, the state capital.

In urban settings with highly mobile populations, studies have suggested that identical IS*6110* RFLP patterns in Mtb isolates from epidemiologically linked patients reflect TB resulting from recent transmission.[5,6] Strains with IS*6110* RFLP patterns that are identical or similar are said to belong to a cluster and represent recent transmission. In 2007, Lazzarini et al. reported the identification of Mtb strains from patients in Rio de Janeiro, Brazil that were characterized by a deletion of a 26.3-kb region that included 10 genes and was referred to as RD^{Rio}. It occurred exclusively among strains of the Latin American–Mediterranean (LAM) spoligotype family.[7–9] The high prevalence in Rio de Janeiro of this RD^{Rio} lineage and its association with more severe disease and cavitary lung lesions suggested enhanced virulence and transmission of this lineage, and therefore its high local prevalence.[7–9]

However, previous studies have not demonstrated whether TB caused by RD^{Rio} strains is more likely to result from recent transmission or reactivation of an old infection.[7–9] The RD^{Rio} designation is based on a multiplex PCR-based test.[7,9] Strains given this designation actually belong to multiple genotypes by IS*6110* RFLP analysis. The present study was therefore undertaken to investigate whether RD^{Rio} strains in Vitória are any more likely than non-RD^{Rio} strains to represent recent transmission by IS*6110* RFLP analysis. If the proportion of RD^{Rio} strains belonging to cluster RFLP patterns is similar to that for non-RD^{Rio} strains, then the high prevalence of RD^{Rio} strains may represent recent introduction of these strains into the community and not necessarily their enhanced virulence.

2. Materials and methods

2.1. Study design and patient data

This was a retrospective, laboratory-based surveillance study of all new TB cases diagnosed in the metropolitan area of Vitória between 2000 and 2010. The metropolitan area comprises four municipalities (Vitória, Cariacica, Serra and Vila Velha) with about 1,200,000 inhabitants.

General epidemiologic characteristics including gender, age, geographic origin, HIV status, previous history of TB and drug-susceptibility profile were collected from laboratory records or medical files and also from the national surveillance system (SINAN).

Countries which have financial resources recommend performing drug-susceptibility testing (DST) for all patients at diagnosis. [10] Countries with limited resources, such as Brazil, do not follow this practice and DST is only recommended for special cases, such as retreatment after failure, relapse, patients with suspected primary resistance and case contacts of resistant tuberculosis.[11] In this study, we have only reported the DST results of the isolates of those patients who had this test requested.

2.2. IS6110 restriction fragment length polymorphism (RFLP) analysis

Genomic DNA isolation and P*vu*II IS*6110* RFLP analysis was performed according to standardized methods.[12]

The IS*6110* RFLP band patterns were analyzed by the Bio-Numerics software version 6.5 (Applied Maths – Belgium). A dendrogram was constructed to show the degree of similarity among the isolates by un-weighted pair group method of arithmetic average (UPGMA) and the Dice index (1.0% tolerance, 1.5% optimization). The analysis was done in 3-year and 11-year windows. Two or more strains with indistinguishable RFLP patterns (fingerprint) were defined to belong to the same RFLP cluster. Strains with RFLP patterns shown to be at least 70% similar were defined to belong to the same family.

Simpson's diversity index was used to calculate the relative frequencies of different number of IS*6110* RFLP genotypes in RD^{Rio} and non-RD^{Rio} groups.[13]

2.3. Spoligotyping

All Mtb isolates were also submitted to spoligotyping analysis by a commercial kit (Ocimum Biosolutions Inc., India) according to a standard protocol.[14,15] The results were recorded in a 43-digit binary format representing the 43 spacers. The spoligotyping patterns were compared with an updated SpolDB4[15] database – SITVIT WEB[16] of Pasteur Institute of Guadeloupe (http//:www.pasteur-guadeloupe.fr: 8081/SITVIT_ONLINE) that provides information on the Mtb spoligotypes worldwide. The orphan patterns were analyzed by the algorithm, SPOTCLUST,[17] to identify the probability of a strain to belong to spoligotype families, based on their spoligotyping patterns.

2.4. Long sequence polymorphism (LSP)

A multiplex PCR adapted from Gibson et al.[18] was performed to differentiate isolates belonging to the RD^{Rio} lineage. The PCR reaction was performed in a final volume of 25 μL, containing 20 pmol of primers BridgeF: 5′ – CAC TCC GGC TGC CAA TCT CGT C – 3′, BridgeR: 5′ – CAC CGC GAG GCT GAA TGA GAC CA – 3′, IS1561F: 5′ – GAC CTG ACG CCG CTG ACA C –3′, IS1561R: 5′ – CAC CTA CAC CGC TTC CTG CC – 3′; 1U Taq polymerase (Invitrogen Life Technologies, USA), buffer 1X, $MgCl_2$ $MgCl_2$ 2.0 mM, DMSO 5%, dNTP 0.2 mM deionized water and 20 ng of genomic DNA. The amplification was done in a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, USA). 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. The PCR products were detected in 1.5% agarose gel treated with ethidium bromide, under UV transillumination. The identification of RD^{Rio} or non-RD^{Rio} strains genotypes was established according to a PCR product band size; the presence of a band of 1175 bp indicated RD^{Rio} and a band of 530 bp indicated non-RD^{Rio} strains.

2.5. Statistics analysis

Covariates were divided into two subgroups: demographic factors (e.g., age, race/ethnicity, gender, educational level) and clinical factors (e.g., symptoms and existing medical conditions). Odds ratios (OR) along with the 95% confidence intervals (CI) and *p*-value

were estimated. In addition, univariate logistic regression was performed for each variable of interest and OR with a *p*-value <0.10 were included in the multivariate logistic regression for calculation of adjusted odds ratios for clustered isolates. Two models were created to show differences between variables. In the first model, the outcome was set up with clustered isolates; in the second model, the outcome was set up with RD^{Rio} genotypes. All analyses were conducted with the Stata® statistical package, Version 9 (StataCorp, College Station, TX, 2001).

3. Results

Between January 2000 and December 2010, 5470 TB patients were diagnosed in the metropolitan area of Vitória. Here, only patients with a positive culture and with bacteriological confirmation for Mtb were included in this study. Good quality RFLP patterns were obtained from isolates from 981 patients (Figure 1).

Demographic, clinical and diagnostic characteristics for all patients are summarized in Table 1 and stratified by RFLP cluster pattern association. The median age of participants was 36 years and the majority was male and non-White. There was no difference between RFLP cluster and non-cluster isolates related to gender, race, educational level, residence and age ($p > 0.05$).

The results of RFLP analysis in the 3-year and 11-year windows are shown in Figure 2. DST was done for 338 isolates. Among these, 13 (7%) of 180 clustered isolates were resistant to at least one drug and; while 17 (11%) of 158 non-clustered isolates were resistant to at least one drug ($p = 0.2$, Chi-square test). The RD^{Rio} analysis showed that 13 (10%) of 128 RD^{Rio} isolates were resistant to at least one drug and; while 17 (8%) of 203 non-RD^{Rio} isolates were resistant to at least one drug ($p = 0.58$, Chi-square test). Of 30 resistant isolates, 7 (2%) were multidrug resistant (MDR); 2 (0.6%) of them were RD^{Rio} and 5 (1.4%) were non-RD^{Rio} genotype.

We obtained chest X-ray information from 878 (90%) of the cases; 865 (99%) had pulmonary TB. Of these, 377 (44%) and 488 (56%) were infected with Mtb strains belonging to an RFLP cluster and a non-cluster pattern, respectively ($p = 0.38$). Among the 681 acid-fast bacilli (AFB) sputum smear test-positive patients, 312 (46%) and 369 (54%) belonged to cluster patterns, while among 215 sputum smear test-negative patients, 80 (37%) and 135 (63%) were infected with Mtb strains belonging to a cluster and a non-cluster pattern, respectively (OR = 1.42; 95% CI: 1.03–1.98; $p = 0.026$).

Multivariate analysis revealed that only RD^{Rio} genotype strains (OR_{adj} 1.52; 95% CI: 1.12–2.05) and Mtb isolates from smear-positive sputum samples (OR_{adj} 1.50; 95% CI: 1.01–2.21) were significantly likely to belong to RFLP clusters.

Among 981 isolates, 375 (38%) were RD^{Rio} and 594 (61%) were non-RD^{Rio} strains; 12 (1%) isolates had two bands by multiplex PCR, indicating a mixed population. Of the 375 RD^{Rio} and 594 non-RD^{Rio} isolates, 180 (48%) and 235 (40%) belonged to an RFLP cluster, respectively ($p = 0.022$, Chi-square test; OR = 1.35, 95% CI:1.04–1.77) as shown in Table 2. We found in the univariate analysis that patients infected with RD^{Rio} were more likely to have pulmonary disease (OR = 2.75; 95% CI: 1.63–4.83; $p = 0.0001$).

Multivariate analysis revealed that clustered isolates (OR_{adj} 1.46; 95% CI: 1.12–1.90) and pulmonary disease (OR_{adj} 2.9; 95% CI: 1.74–4.85) were significantly associated with RD^{Rio} genotype. Genotyping by IS*6110* RFLP showed that the 180 RD^{Rio} isolates in clusters belonged to 45 distinct IS*6110* RFLP genotypes while the 235 non-RD^{Rio} clustered isolates

were comprised of 75 distinct genotypes ($p = 0.30$). Simpson's diversity index for RD^{Rio} was 0.96 and for non-RD^{Rio} was 0.98, indicating great diversity in both groups.

Spoligotype data were obtained from 619 isolates (63%) (Supplementary Figure 1); 530 could be defined to the SIT level while 89 had orphan patterns. In addition, 384 (62%) were of the non-RD^{Rio} genotype, 225 (36%) of the RD^{Rio} genotype and 10 (2%) were of mixed genotypes, similar to the distribution found with the RFLP analysis. Spoligotyping family and SIT information are presented in Table 3. We also observed RD^{Rio} among 2 isolates defined as T (1%), 5 as Haarlem (2%), 6 (3%) as unknown families and 20 orphan genotypes (9%).

4. Discussion

Several studies have reported the use of genotyping tests to characterize bacterial population structure of Mtb strains and their transmission in defined communities. A high prevalence of TB caused by identical or related Mtb lineages in a community could represent recent introduction or enhanced virulence of such strains, or some epidemiologic factors that facilitate increased transmission of such strains. Whether a genetic makeup of Mtb strains is a determinant of TB transmission or severity of disease is often difficult to assess because of limited clinical data.[19]

Lazzarini et al. in 2007 described Mtb RD^{Rio} genotype as a clonally-derived sublineage within the LAM family, characterized by a deletion of a 26.3-kb region that included 10 genes.[7] The Mtb RD^{Rio} sublineage is the most prevalent cause of TB in Rio de Janeiro and is also present in other Brazilian and world regions[7–9] The high prevalence in Rio de Janeiro of this RD^{Rio} lineage and its association with more severe disease and cavitary lung lesions suggested enhanced virulence of strains belonging to this lineage.[7–9]

As far as we know, this study in Espírito Santo is the first population-based study to determine the incidence of RD^{Rio} in a Brazilian population and the largest number of strains analyzed by RFLP typing in a single study in Brazil. The prevalence of TB caused by Mtb RD^{Rio} in Espírito Santo was found to be 38%, similar to other studies. However, the other studies were based on convenience clinical isolates collected in hospitals and TB reference laboratories.[8]

In contrast to most studies done in Brazil, our study found that 6% of the RD^{Rio} isolates belonged to spoligotyping families other than LAM and this was also reported by Weisenberg et al.[9]

In the univariate and multivariate analysis, RD^{Rio} genotype strains were significantly more likely to belong to RFLP cluster patterns than non-RD^{Rio} strains, indicating TB caused by RD^{Rio} were more likely to represent recent transmission. Isolates from AFB smear-positive patients were also more likely to belong to RFLP cluster patterns. Several studies have demonstrated that patients who have cavitary lung disease or positive AFB smears in sputum are indeed high transmitters, and hence such strains may be more overly represented in communities.[20,21]

Studies published after 1999 have generally used more than one genotyping method to demonstrate clustering.[22–24] The use of only one high-resolution typing method (IS*6110* RFLP) for a subset of the samples is one limitation of our present study.

Nevertheless, the findings of the analysis of RFLP in this study showed that the percentage of clustering is dependent on the number of Mtb isolates analyzed. The proportion of clustering increases as the number of isolates in each year window period increases, as

reported by van Soolingen et al.[25] As seen in Figure 2, the proportion of strains with clustered RFLP patterns during the period (2003–2005) with the highest number (518) of isolates was similar to that found for all the isolates (981) obtained during the entire 11-year period.

The fact that we found a greater proportion RD^{Rio} genotypes to belong to RFLP cluster patterns could mean the following: (i) that the RD^{Rio} strains are more biologically "fit", as suggested by Lazzarini et al.,[8] or (ii) that these strains were recently introduced into this region of Brazil and evolved after the introduction. RD^{Rio} was previously reported to be associated with more severe TB,[8] but in our study, we observed that it was less likely to cause extrapulmonary disease than non-RD^{Rio} strains.

Although the proportion of cluster patterns among RD^{Rio} strains (48%) was significantly greater than that among non-RD^{Rio} strains (40%), it is not clear if this difference could be attributed to enhanced virulence or transmissibility of the RD^{Rio} strains. The RFLP genotypes showed a high level of diversity in both RD^{Rio} (Simpson's diversity index = 0.96) and non-RD^{Rio} strains (Simpson's diversity index = 0.98), indicating both groups of Mtb strains were highly heterogeneous. Most of the isolates included in the previous study in Rio de Janeiro were obtained from patients with clinically suspected "complicated" TB, advanced disease, or associated comorbidities, which might have contributed to the association of RD^{Rio} genotypes with a more "severe" disease.[8] From a public health and TB control perspective, if 40–48% of new TB cases in a community are resulting from recent transmission, the new cases that may be attributed some biologic factors (possibly 8%) appear to be quite small. The priority of TB control program in Espírito Santo should emphasize programs to identify epidemiologic factors that facilitate enhanced transmission so that such factors could be eliminated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.tube.2012.09.001.

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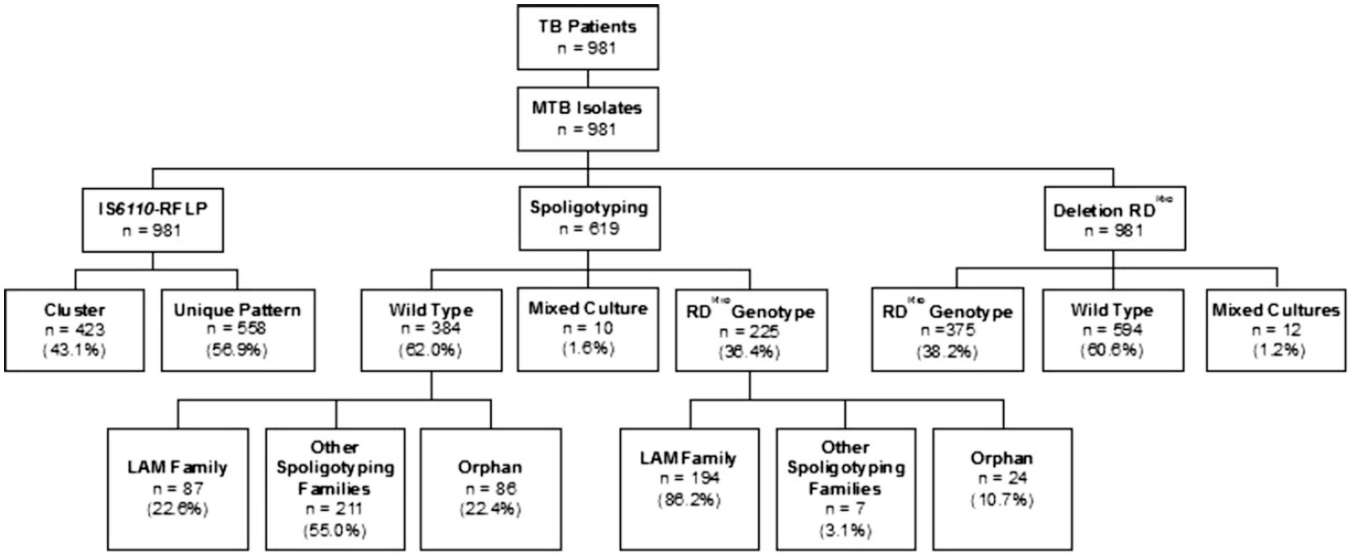


Figure 1.
Flow chart of number of isolates and genotyping methods.

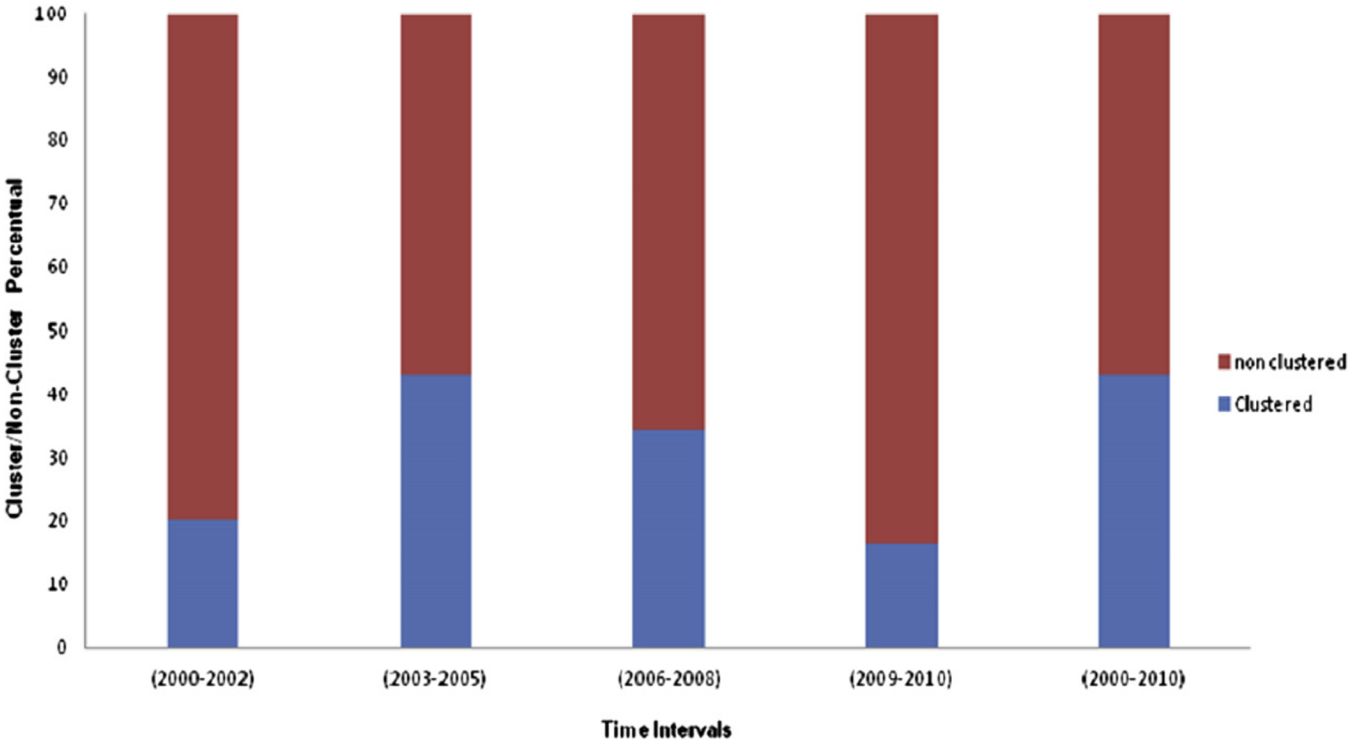


Figure 2.
Percentage of RFLP type clustering of isolates at differential time intervals.

Table 1

Demographic and clinical characteristics of TB patients according to their Mtb isolates' IS6110 RFLP cluster patterns in the metropolitan area of Vitória, Brazil, 2000–2010.

	Clustered isolates (%)	Non-clustered isolates (%)	OR (95% CI)	*p*-value
Gender				
Female	125 (43%)	169 (57%)	0.97 (0.72–1.28)	0.81
Male	298 (43%)	389 (57%)		
Race				
White	82 (40%)	123 (60%)	0.81 (0.57–1.15)	0.81
Non-White	214 (45%)	261 (55%)		
Municipality				
Vitória city	267 (45%)	332 (55%)	1.18 (0.90–1.55)	0.21
Other cities	153 (40%)	225 (60%)		
Age				
Mean (SD)	36.17 (13.85)	37.61 (13.87)	-	0.11
TST				
Positive = 10 mm	365 (50%)	370 (50%)	0.89 (0.50–1.57)	0.67
Negative <10 mm	31 (52%)	28 (48%)		
HIV				
Positive	45 (41%)	65 (59%)	0.91 (0.59–1.40)	0.19
Negative	303 (43%)	400 (57%)		
TB presentation				
Pulmonary	377 (44%)	488 (56%)	1.25 (0.77–1.89)	0.38
Extra-pulmonary	39 (39%)	61 (61%)		
Smear results				
Positive	312 (46%)	369 (54%)	1.42 (1.03–1.98)	0.026
Negative	80 (37%)	135 (63%)		
Genotype				
RD^{Rio}	180 (48%)	196 (52%)	1.35 (1.04–1.77)	0.02
Non-RD^{Rio}	235 (40%)	358 (60%)		

OR = odds ratio; CI = confidence interval.

Table 2

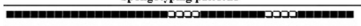
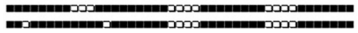
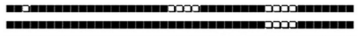
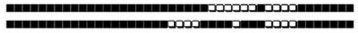
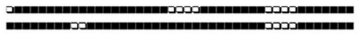
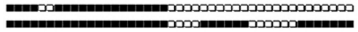
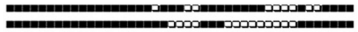
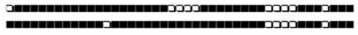
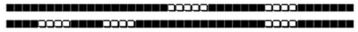
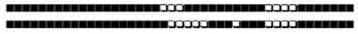
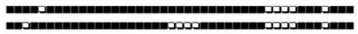
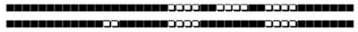
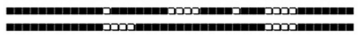
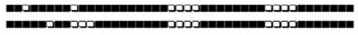
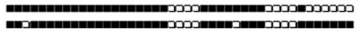
Demographic and clinical characteristics of patients infected with RD^{Rio} or non- RD^{Rio} Mtb strains in the metropolitan area of Vitória, Brazil, 2000–2010.

	RD^{Rio} isolates (%)	**Non-RD^{Rio} isolates (%)**	**OR (95% CI)**	***p*-value**
Gender				
Female	117 (40%)	176 (60%)	1.07 (0.81–1.44)	0.59
Male	258 (38%)	419 (62%)		
Race				
White	73 (36%)	131 (64%)	0.85 (0.61–1.19)	0.34
non-White	302 (39%)	464 (61%)		
Municipality				
Vitória	231 (39%)	363 (61%)	1.02 (0.77–1.34)	0.85
Other cities	144 (38%)	232 (62%)		
Age				
Mean (SD)	36.7 (13.35)	371 (14.26)	-	0.68
TST				
Positive = 10 mm	365 (50%)	370 (50%)	0.89 (0.50–1.57)	0.67
Negative <10 mm	31 (53%)	28 (47%)		
HIV				
Positive	37 (35%)	70 (65%)	0.83 (0.53–1.29)	0.40
Negative	271 (39%)	427 (61%)		
TB presentation				
Pulmonary	351 (41%)	503 (59%)	2.75 (1.63–4.83)	0.0001
Extra-pulmonary	20 (20%)	79 (80%)		
Smear results				
Positive	264 (39%)	409 (61%)	1.04 (0.75–1.45)	0.79
Negative	81 (38%)	131 (62%)		
RFLP				
Clustered isolates	180 (43%)	235 (57%)	1.35 (1.04–1.77)	0.022
Non-clustered isolates	196 (35%)	358 (65%)		

OR = odds ratio; CI = confidence interval.

Table 3

Spoligotyping patterns and frequency of Shared International Type (SIT).

Spoligotyping patterns	SIT	Family	Frequency
	42	LAM9	76
	33	LAM3	39
	17	LAM2	38
	20	LAM1	30
	53	T1	28
	47	H1	20
	64	LAM6	17
	50	H3	16
	60	LAM4	16
	177	LAM9	16
	34	S	13
	2449	Ambiguous LAM5 LAM4	10
	1241	LAM9	9
	167	T1	9
	119	X1	9
	137	X2	8
	93	LAM5	7
	95	LAM6	6
	39	T4-CEU	6
	2110	H3	5
	828	LAM4	5
	73	Ambiguous T3 T2	4
	230	LAM9	4
	1284	T1	4
	1051	T1	4
	228	T1	4
	153	T2	4
	1321	Ambiguous LAM1 LAM4	3
	59	LAM11-ZWE	3
	216	LAM5	3
	176	LAM6	3
	102	T	3
	306	T1	3
	291	T1	3
	92	X3	3
	62	H1	2
	741	H3	2
	2536	LAM1	2
	2646	LAM3	2
	1106	LAM4	2
	1755	LAM6	2
	1758	LAM9	2
	51	T1	2
	1227	T5_MAD2	2
	58	T5-MAD2	2
	439	X2	2
	450	Ambiguous X1 T5	1
	1	Beijing	1
	1983	EAI3-IND	1
	571	H1	1
	382	H1	1
	218	H1	1
	2	H2	1
	746	H3	1
	36	H3	1
	2654	LAM	1
	2566	LAM1	1
	2271	LAM2	1
	194	LAM2	1
	179	LAM2	1
	2388	LAM3	1
	1841	LAM3	1
	211	LAM3	1
	163	LAM4	1
	1337	LAM5	1
	1610	LAM6	1
	1815	LAM8	1
	2165	LAM9	1
	1154	LAM9	1
	866	LAM9	1
	162	LAM9	1
	2025	T	1
	1905	T1	1
	1144	T1	1
	1122	T1	1
	462	T1	1
	122	T1	1
	1660	T2	1
	233	T2	1
	175	T2	1
	52	T2	1
		Total	488