Typing of *Mycobacterium tuberculosis* strains isolated in Community Health Centers of Rio de Janeiro City, Brazil

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Fingerprinting of Mycobacterium tuberculosis *strains from tuberculosis (TB) patients attended in Community Health Centers (CHCs) of Rio de Janeiro was performed to verify possible risk factors for TB transmission. A prospective community-based study was performed during the period of July 1996 to December 1996 by collecting sputum samples of 489 patients in 11 different CHCs in four different planning areas (APs) of the city. Bacteriological, clinical, and epidemiological information was collected and* M. tuberculosis *genotypes defined after restriction fragment length polymorphism (IS*6110*-RFLP) and double repetitive element (DRE) fingerprinting of RFLP-clustered cases. Risk factors for TB transmission were looked for using three levels of cluster stringency. Among 349 (71%) positive cultures obtained, IS*6110*-RFLP typing could be performed on strains from 153 different patients. When using identity of RFLP patterns as cluster definition, 49 (32%) of the strains belonged to a cluster and none of the clinical or epidemiologic characteristics was associated with higher clustering levels. However, higher clustering level was observed in the AP including the central region of the city when compared to others. This strongly suggests that more recent transmission occurs in that area and this may be related with higher incidence of TB and HIV in this region.*

Key words: tuberculosis - molecular epidemiology - IS*6110*-restriction fragment length polymorphism - double repetitive element

It is generally accepted that one third of the world population is infected with *Mycobacterium tuberculosis* (Mtb), and the estimated mortality because of tuberculosis (TB) in 2004 was nearly 2 million, demonstrating that Mtb is among the most deadly human infectious agents. The majority (95%) of TB cases occur in developing countries, a scenario mainly responsible for negligence of the seriousness of the disease in richer countries. The latter however were obliged to reconsider the importance of the disease, due to the association between increased TB risk and HIV infection and the spreading of multi-drug resistant (MDR) strains all over the world (WHO 2006).

In developed nations, epidemiologic investigations that incorporate genotyping of Mtb by restriction fragment length polymorphism (RFLP) have lead to novel information about the spread of Mtb in the community and during institutional outbreaks, to a better understanding of the transmission dynamics of TB and to the differentiation of (re)infection from reactivation (Barnes & Cave 2003, Seidler et al. 2004).

Since the early nineties, definition of risk factors for TB transmission in high TB incidence areas has been complemented by information obtained by Mtb genotyping, the latter based on the recognition that clusters of fingerprints are a measure of recent transmission (Palittapongarnpim et al. 1997, Pineda-Garcia et al. 1997, Dale et al. 1999, Haas et al. 1999, Fandinho et al. 2000, Ferrazoli et al. 2000, Bruchfeld et al. 2002, Ferdinand et al. 2003, Easterbrook et al. 2004, Das et al. 2005). Mostly however, a convenient, and not a population-based and therefore adequate sampling, has been submitted to genotyping (Wilkinson et al. 1997, Van Rie et al. 1999, Godfrey-Faussett et al. 2000, Narayanan et al. 2002, Chan-Yeung 2003, Verver et al. 2004). Nonetheless, adding genotyping data, to those obtained during more traditional epidemiologic approaches, has revealed that particular Mtb strains can be responsible for MDR-outbreaks in the community, can disseminate more quickly or induce more severe forms of TB and can be responsible for failure or relapse during and after treatment (Lan et al. 2003).

In Brazil, TB remains an important public health problem, the country ranking 16th among the 22 countries with the highest TB burden worldwide. These high-burden countries account for approximately 80% of the estimated number of new TB cases (all forms) arising each year (WHO 2006). Together with Peru, Brazil accounted for 50% of all TB cases in Latin America in 2002. The high prevalence of TB in Southeast Brazil is presumed to be a result of worsening living conditions in the urban areas, the HIV epidemic, inadequate diagnosis and treatment and inadequate use of resources. In this region, Rio de Janeiro, the countries' second largest city, presents approximately 9000 new cases per year, an average incidence rate of 175/100,000 (1998-2002) and a mortality rate of 12 per 100,000 inhabitants, among the high-

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est rates in the country (Cavalcante et al. 2003). Some strategies to revert this picture were induced, including the use of Directly Observed Therapy, Short-Course (DOTS) TB strategy in 1998 and provision of free antiretroviral therapy (HAART) for all diagnosed HIV cases in 1997 (Ministry of Health BNSAp. 2004, WHO 2005).

To our knowledge, only one study on the contribution of Mtb genotyping to evaluate TB detection of transmission, in the general population in Brazil, has been reported, namely in the City of São Paulo (Telles et al. 2005). We here present the use of IS*6110*-RFLP typing and DRE-PCR for cluster confirmation in an attempt to estimate the risk factors and regions for transmission of TB in the city of Rio de Janeiro, in a period before the DOTS and HAART implementation.

MATERIALS AND METHODS

Study location and population - Diagnosis and treatment of patients within the TB control program of Rio de Janeiro, a city with 5.6 million inhabitants in 1996, occurs in 28 Community Health Centers (CHCs), 48 general hospitals, two specialized hospitals for TB treatment, and two prisons. These institutions are localized in different areas of the city, divided into ten major planning areas (AP; Fig. 1), each covered by at least two CHCs, responsible for attending persons with suspicion of TB that are usually living or working in that region. Annually, 6000 TB cases are attended in CHCs and 3300 in hospitals or prisons. The number of individuals included in the bacteriogical evaluation for each CHCs/AP was convenient and not strictly population-based.

From July to December 1996, individuals older than 14 years with suspicion of TB, and being attended at one of the 11 CHCs of AP1, AP2, AP3 or AP5 were considered for inclusion in the study, independent of sex, race, socioeconomic class, and HIV status. TB was diagnosed according to recommendations by WHO (2005) and at time of diagnosis, after written consent, a blood sample and two consecutive sputum samples were collected. The blood samples were stored at –20^{o}C until further analysis. An individual was considered a TB patient when positive culture for Mtb or two sputum smears positive for acid-fast bacilli (AFB+) were obtained. Smear negative pulmonary TB cases had at least two smear examinations negative for AFB, radiographic abnormalities consistent with active pulmonary TB, no response to a course of broad-spectrum antibiotics, decision by a clinician to treat with a full course of anti-TB therapy, or positive culture but negative AFB sputum examinations.

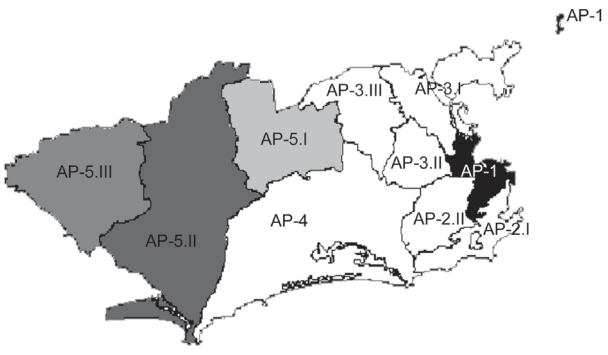

Fig. 1: map of the City of Rio de Janeiro with the different "Programatic Areas" for tuberculosis control.

The site coordinator was responsible for collecting demographic, epidemiological, clinical, and laboratory data. Subjects were asked to answer a standardized questionnaire that comprised information regarding potential risk factors for TB infection such as sexual preference, living conditions (including slums), attendance at a hospital, education level, sex, permanence in a prison or police station, clinical form of TB, earlier treatment, intravenous drug use, drug resistance, HIV infection, and presence of a BCG scar. All clinical samples were sent to the Central Laboratory of Health Noel Nutels (Lacen, RJ) for laboratory analysis.

This study was approved by the Institutional Review Boards of the Clementino Fraga Filho University Hospital of the Federal University of Rio de Janeiro and by those of each participating institution.

Specimen collection, culture collection, diagnostics, and drug sensitivity testing - Infection with HIV-1 and HIV-2 was verified by serology (Organon Teknica, Boxtei, The Netherlands) and positive cases were confirmed by Western blotting (Dupont, Wilmington, DE, US). Sputum was processed using the acetyl-cysteine method and samples were submitted to bacilloscopy according to Ziehl-Nielsen and culture in Löwenstein-Jensen medium (L-J), following the recommendations of the Brazilian National TB Control Program (Brasil 1994). Positive cultures were submitted to standard identification procedures for differentiation of species belonging to the Mtb Complex (MTBC) from atypical mycobacteria (Kent & Kubica 1985). Isolates were tested for susceptibility to isoniazid, rifampin, ethambutol, pyrazinamide, and streptomycin, using the proportion method (Brasil 1994, Kent & Kubica 1985).

DNA fingerprinting - Cultures on L-J were submitted to DNA extraction and IS*6110*-RFLP typing according to a standardized methodology as described by van Embden et al. (1993). In each gel and each sample respectively, DNA from the reference strain Mt14323 and internal molecular weights markers for IS*6110*-containing fragment position normalization were included. The RFLP patterns were introduced into the GelCompar® software (Windows version 4.0; Applied Maths, Kortrijk, Belgium) and a similarity matrix and dendrogram were constructed using the Dice similarity coefficient and the UPGMA algorithm, using a position tolerance of 1.2%. Clusters of IS*6110*-RFLP, indicated by GelCompar, were visually verified for consistency.

The majority of samples characterized by IS*6110*-RFLP were also submitted to "Double Repetitive Element" PCR (DRE-PCR) fingerprinting as described by Montoro et al. (1998). Briefly, 2 µl of each sample was added to a PCR mixture containing 20 mM of each dNTP, 6% dimethyl sulfoxide, 1.25 U Taq polymerase, and 100 ng of primers Ris1, Ris2, Pntb1, and Pntb2 in a total volume of 50 µl. The samples were amplified by thermal cycling during 1 min at 95°C, 2 min at 56°C, and 1 min

at 72°C and a final cycle of 7 min at 72°C. Amplified products were verified by gel electrophoresis in 2% agarose and ethidium bromide staining and a 50 bp ladder was added to each gel as molecular weight marker.

Cluster definition - For cluster definition, three different stringencies were applied, including, ranging from more to less stringent: (i) identical IS*6110*-RFLP and DRE patterns, (ii) identical IS*6110*-RFLP patterns, and (iii) IS*6110*-RFLP patterns with 90% similarity on the dendrogram. Due to the sampling characteristics and typing efficiency in the present study, we applied the n-method for cluster degree calculation (Murray & Alland 2002).

Statistical analysis - Data were analyzed using Epi Info (version 6.03, CDC, Atlanta, GA, US; public domain). Statistical significance of levels of association of cluster and non-cluster with epidemiologic and demographic characteristics for categorical variables were compared by the Fisher exact or chi-squared test. Continuous variables were compared by the Student t-test or the Kruskal-Wallis rank-sum test. A confidence internal (CI) of 95% was used in all odds ratio (OR) calculations.

RESULTS

Patients and specimen collection - From July to December 1996, 859 TB cases were attended in 11 CHCs. Excluded from further analysis were 295 (34%) cases that did not live in Rio de Janeiro, 67 (7.8%) patients that had extrapulmonary TB, 48 (5.5%) individuals that refused to participate in the study, and 26 (3%) cases that had been using anti-TB drugs for more than 15 days. No statistically significant difference was observed regarding sex, age, and history of anti-TB treatment between included or excluded cases (data not shown). Sputum samples were collected from 438 patients (51%) with available clinical, demographic, and epidemiological data. Serology for HIV was performed in 309 of these patients; positive results were confirmed in 27 cases (8.7%) and a higher proportion of HIV positivity (12.3%) observed in AP1 in comparison to other APs (median of 8.2%).

Cultures and drug susceptibility patterns - Positive cultures for *Mycobacterium* were obtained from 349 different patients (82%) and all were identified as belonging to the MTBC. Among these, 236 strains were submitted to susceptibility testing; the proportions of primary and acquired drug resistance were respectively 7.1 and 24.1%. MDR, as defined by resistance to at least INH and RIF was detected in isolates from two patients that had a history of previous TB treatment.

Fingerprinting - Good quality IS*6110*-RFLP patterns were obtained from cultures of 153 patients, 47% of the culture positives TB cases. As demonstrated in Fig. 2, Mtb genotypes belonged to a limited number of groups as defined by the UPGMA-based dendrogram. Furthermore, genotypes presented between two and 22 copies of IS*6110* with the majority (56%) having between eight and ten copies; only two strains had less than six IS*6110* copies. Forty nine of the isolates (32.7%) presented a pattern that belonged to one of 21 clusters, including one cluster containing five isolates, five with three, and 15 with two samples.

Upon decreasing stringency of cluster definition to 90% similarity of RFLP patterns in the dendrogram, 28 clusters containing 72 strains (45.1%) were observed. Among strains submitted to RFLP, DRE-PCR typing could be performed on 136 (data not shown), including 43 of the 49 (88%) RFLP clustered strains. Among the latter, 34 (79%) also had clustered DRE-PCR profiles but three RFLP clusters of two strains and three strains of the biggest RFLP cluster were discriminated by DRE-PCR. In the RFLP clusters with two strains, in each case, a single band difference was observed in the DRE pattern. Among the DRE patterns observed in strains forming the biggest RFLP cluster, four had a prominent band in common and two of these had an extra band on of different size; the fifth strain had an unrelated pattern (data not shown). The same DNA preparations were used for both techniques so sample mixup is unlikely to be the reason for this observation.

Association of patient and strain characteristics with genotype clustering - The clinical, epidemiological, and demographic characteristics of the patients, strain characteristics and cluster frequency as defined by identical RFLP patterns is presented in Table I. No statistically significant association between cluster level and any of these characteristics was observed. When other stringencies were used for cluster definition, again, no significant association of clustering with any characteristic was observed (data not shown).

To verify whether there was any relationship between the origin of the patients and recent TB transmission level, cluster frequency was determined for each AP separately. Significantly more clustering was observed among strains from patients attended in AP1 when compared to the others when using identical RFLP patterns for cluster definition (Table II). Interestingly, this difference continued statistically significant when increasing stringency of cluster definition (data not shown). To verify whether cluster frequency in the APs was directly related to the fraction of genotyped strains among those from all patients diagnoses in the different APs, both frequencies were compared, and as demonstrated in Table II, no direct relation was observed between both.

DISCUSSION

Investigation of TB transmission employing genotyping of Mtb as an additional tool was initially performed in some large cities of the United States (Small et al. 1994, Munsiff et al. 2003) and Europe (Lambregts-van Weezenbeek et al. 2003). These studies demonstrated the important contribution of recent TB transmission in many new TB cases, even in areas of low incidence, where TB was considered to be a controlled disease. Fingerprinting of Mtb also allowed better recognition of nosocomial and community outbreaks and of unsuspected or intricate chains of transmission (Caminero et al. 2001, Barnes & Cave 2003, Seidler et al. 2004). Genotyping of Mtb strains also led to the visualization of the region-associated population structure of the bug and of differences in strain variability and prevalence, as occurring both on a small and large scale. A considerable number

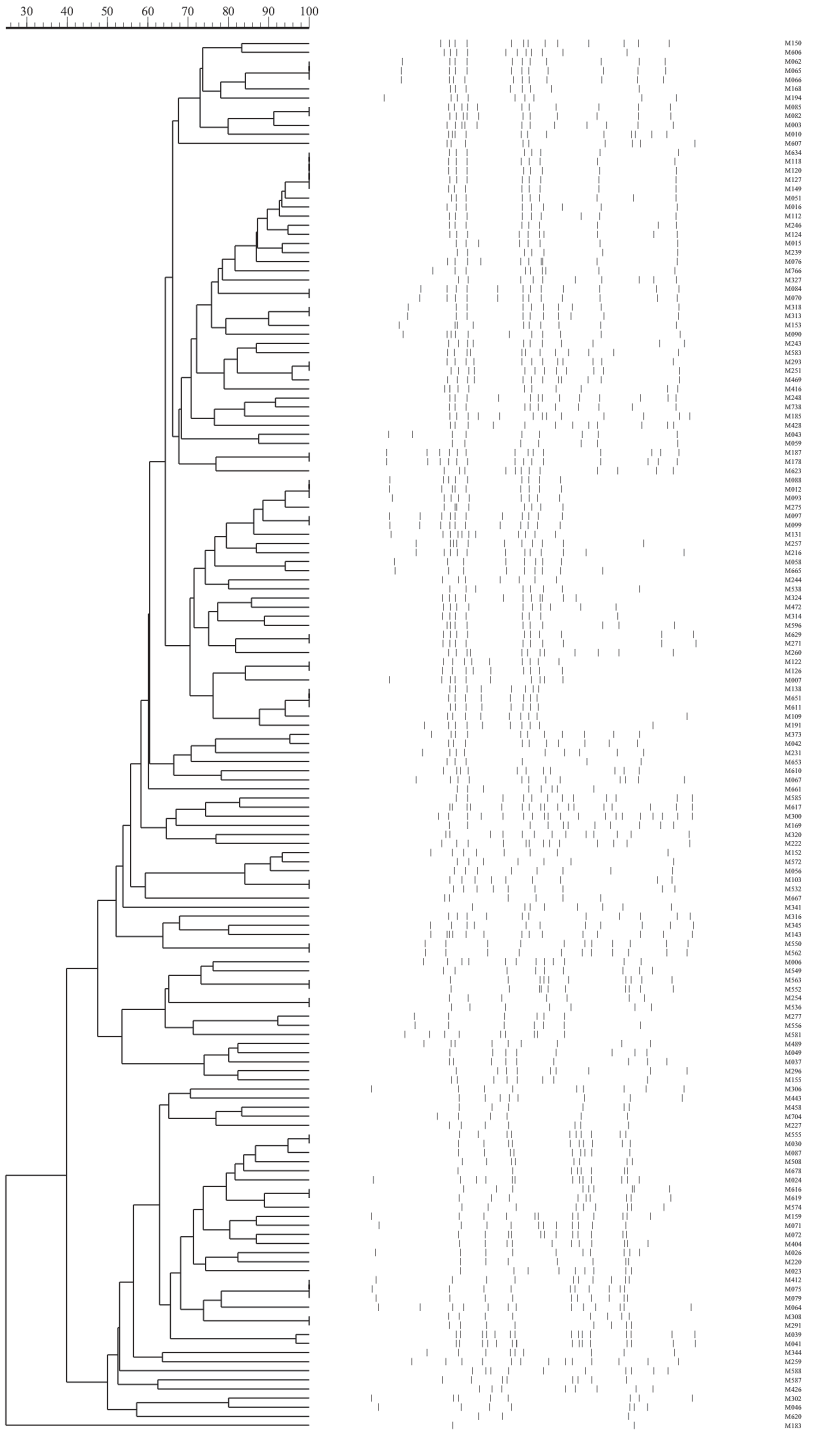

Fig. 2: dendrogram of IS*6110*-restriction fragment length polymorphism patterns obtained from 153 different patients.

of studies were performed on TB cases from developed countries: These observations, in addition to recent data from patients residing in developing countries, including Brazil, demonstrate the need to evaluate locally, both, epidemiologic and Mtb genotyping characteristics (Palittapongarnpim et al. 1997, Pineda-Garcia et al. 1997, Wilkinson et al. 1997, Dale et al. 1999, Haas et al. 1999, Fandinho et al. 2000, Ferrazoli et al. 2000, Godfrey-Faussett et al. 2000, Suffys et al. 2000, Bruchfeld et al. 2002, Narayanan et al. 2002, Ferdinand et al. 2003, Easterbrook et al. 2004, Verver et al. 2004, Das et al. 2005).

To our knowledge, this study is the first population based analysis supported by fingerprint data of TB transmission in patients from Rio de Janeiro. Importantly, the study presents some limitations, including the incomplete analysis of the isolates of the TB cases reported during the sampling. This could have influenced our study outcome because clustering level has been directly as-

TABLE I

Patients and strain characteristics and IS*6110*-restriction fragment length polymorphism clustering (100% identity) of *Mycobacterium tuberculosis* strains from 153 tuberculosis patients (TB)

Possible risk factor	Number of cases		OR	95% CI	*P*-value
	Cluster	Non cluster			
Age (in years)					
< 20	5	9			
20-30	9	26			
31-40	15	26			
41-50	9	23			
> 50	11	21			0.84
Living conditions					
Homeless/Shelter/Precarious (Slums)	31	61			
House/Apartment	12	22	0.93	0.38-2.31	0.97
Sexual preference					
Homosexual/Bisexual	2	3			
Heterosexual	71	76	1.24	0.14-9.61	1.00
Education					
Illiterate	3	9			
Elementary incomplete	33	65			
Elementary complete	6	11			
High school/College	5	4	0.66	0.25-1.74	0.49
Gender					
Male	37	70			
Female	12	34	1.50	0.65-3.48	0.40
Permanence in hospital					
Yes	12	17			
No	32	68	1.50	0.59-3.86	0.48
Permanence in police station or prison					
Yes	6	7			
No	39	80	1.76	0.48-6.35	0.36
Clinical form of TB					
Pulmonary	37	80			
Extrapulmonary	0	2	0.00	0.00-9.24	1.00
Previous TB treatment					
No	37	69			
Yes	8	13	0.87	0.30-2.55	0.98
Intravenous drug abuse					
Yes	2	3			
No	44	87	1.32	0.15-10.19	1.00
Contact with TB					
Yes	27	66			
No	18	20	0.45	0.19-1.06	0.07
Smear positive cases					
Yes	36	73			
No	13	32	1.21	0.53-2.78	0.76
Drug resistance (at least one drug)					
Yes	3	14			
No	37	85	0.49	0.11-1.99	0.34
HIV serology					
Positive	4	8			
Negative	44	87	0.99	0.23-3.90	1.00
BCG scar					
Yes	31	58			
No	14	31	1.18	0.51-2.74	0.81

→

Possible risk factor	Number of cases		OR	95% CI	*P*-value
	Cluster	Non cluster			
Geographic area[a]					
AP1	12	11			
AP2	11	29			
AP3	18	48			
AP5	8	16	2.74	1.02-7.41	0.03

a: AP1 versus AP2 + AP2 + AP3 + AP5.

TABLE II

Distribution of culture positivity and genotyping of *Mycobacterium tuberculosis* isolates from patients attended at the "programatic areas" included in the study

Area	Number of culture positive patients	Number of RFLPs performed: n (%)	Cluster frequency [a] n (%)
AP1	62	23 (37.0)	12 (47.8)
AP2	103	40 (38.8)	11 (27.5)
AP4	123	66 (53.6)	18 (27.2)
AP5	61	24 (39.3)	8 (33.3)
Total	349	153 (47.8)	49 (32.0)

a: as defined by 100% identical RFLP patterns.

sociated with sampling (Glynn et al. 1999, Murray et al. 2002). Fortunately, the clustering levels, observed in the different APs, seem not to be directly related to the sampling fraction size, maintaining our hypothesis that higher clustering in the AP1 region is a measure of more recent transmission. A second limitation could be the time frame of six months for sample collection. In order to avoid missing clustered cases, it has been proposed to collect samples during at least three years study period and to analyze 70% of all TB cases covered (Vynnycky et al. 2003). Such an approach is however very difficult to establish in an environment such as the city of Rio and few studies, even those performed in developed nations, fulfill this criteria (Barnes & Cave 2003). However, as the annual risk of infection in the Rio de Janeiro region had not greatly changed during the last decade (Cavalcante et al. 2003), this study may provide reliable positive predictive values for clusters.

Two main characteristics of the Mtb genotypes observed in the present study, a considerable variability and the presence of few low-copy number strains have been described in earlier Brazilian studies (Fandinho et al. 2000, Ferrazoli et al. 2000, Suffys et al. 2000, Telles et al. 2005, Cafrune et al. 2006). This is in concordance with genotype structure observed in most countries but in contrast with studies performed in the Middle East and Asia (Dale et al. 1999, Narayanan et al. 2002, Das et al. 2005). Also, genotype cluster frequency presently observed is similar to the levels of 31 to 48% described by some investigators from different parts of the world (Wilkinson et al. 1997, Dias et al. 1998, Ferdinand et al. 2003, Das et al. 2005) lower than the 51 to 72% levels reported in India, Turkey, and some African countries (Godfrey-Faussett et al. 2000, Narayanan et al. 2002, Verver et al. 2004) and higher to the 24% mentioned in Hong Kong (Chan-Yeung et al. 2003). Notably, the largest cluster was formed by a particular 8-band genotype that takes part of a large clade with very similar RFLP patterns, observed in an earlier study on Brazilian strains from different regions of the country (Suffys et al. 2000).

The most important finding of this study is that IS*6110*-patterns obtained from strains of patients residing in programmatic area AP1, located in downtown Rio de Janeiro, were significantly associated with clustering. Possibly, poverty, overcrowding, increased human circulation, and higher HIV infection rate among TB registered cases in this area are contributing to recent transmission of TB. This area of the city has traditionally been reported as having higher TB incidence than other parts (Cavalante et al. 2003). Higher clustering, in some specific urban areas with higher TB incidence, has described in other big cities (Verver et al. 2004). Besides residence, no other case or parasite characteristics were associated with genotype clustering. Not all epidemiologic studies combining conventional and molecular approaches identify risk factors for TB transmission but among those frequently reported are sputum smear positivity (Verver et al. 2004), history of imprisonment or hospitalization (Narayanan et al. 2002), relapse (Narayanan et al. 2002), failure of anti-TB treatment (Godfrey-Faussett et al. 2000), young age, and belonging to social networks. As related before, besides the absence of risk factors being due to sampling, differences in regional (geographic, whether), population (ethnic, behavioral), and bacterial population-associated characteristics also influence transmission patterns; indeed, some assumptions on the disease may be true only in a particular environment (Cohn et al. 1998). Indeed, HIV-infection, drug-resistant forms of TB and homelessness, factors often associated with more intense TB transmission in developed nations, does not seem to influence clustering as much in poor nations. In the latter, and supporting our data, clustering tended to occur more frequently among isolates from patients residing in areas where TB incidence is higher (Small et al. 1994, Barnes & Cave 2003, Lambregts-van Weezen-beek et al. 2003, Munsiff et al. 2003, Seidler et al. 2004).

Although differences in Mtb genotype clustering is the basis for definition of risk factors for TB, confirmation of transmission between patients belonging to clusters enforces the value of this statement. In the present evaluation, this could not be performed due to the design of the questionnaire and lack of funds for re-interviewing selected cases but in studies where this was performed, an epidemiologic link ranging from 16 to 30% was observed among the clustered cases, demonstrating the gain in information when using Mtb genotyping but suggesting also the need to better investigate the accuracy of the procedure in general (Wilkinson et al. 1997, Montoro et al. 1998, Chan-Yeung et al. 2003, Das et al. 2005).

Cluster level, observed during molecular epidemiologic studies, is dependent on the stringency of analysis: small changes of IS*6110* banding patterns occur during transmission and can lead to underestimation of clustering levels and missing transmission chains or cases (Warren et al. 2002). Lowering cluster definition stringency did not influence the outcome of the present study. Nonetheless, many reports including molecular data for TB studies use a secondary typing to increase the accuracy of indicating epidemiological links. In the case of IS*6110*-RFLP patterns, this is considered necessary mainly, when dealing with Mtb populations presenting a high proportion of low-copy number strains (Rhee et al. 2000). Besides, data exist on increased accuracy of typing data when using a secondary technique upon interpretation of IS*6110*-RFLP clusters even of high copy number patterns (Yang et al. 2001). When considering only strains with identical IS*6110*-RFLP and DRE-PCR patterns as part of a cluster (85% of analyzed IS*6110*-RFLP clustered strains), the significantly higher cluster level in AP1 was maintained. Differentiation by DRE-PCR of strains with clustered IS*6110*-RFLP patterns was generally due to single band differences and could be related to the presence of a sub-population detected by PCR but not by Southern-based techniques. The difference between DRE-PCR patterns of some of the strains forming the biggest RFLP cluster was more pronounced but we suspect that this RFLP pattern, besides being also the most frequent in some other studies on Brazilian Mtb genotypes, represents a dominant and deeply rooted clade and on its own does not always indicate recent transmission (Suffys et al. 2000). This is now under investigation.

Concluding, limitations in sampling is probably a main factor for lack of definition of risk factors for TB transmission in the present study but the Mtb fingerprinting data are in agreement with the indication of the central part of Rio de Janeiro being a more problematic area of TB. This encourages the use of fingerprinting in this setting in a well designed prospective epidemiologic study, as being realized for evaluation of the DOTS program from the City TB Program, implemented in this area in 1998 (Cavalcante et al. 2003).

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