Drug Resistance and Genotypes of Strains of Mycobacterium tuberculosis Isolated from Human Immunodeficiency Virus-infected and Non-infected Tuberculosis Patients in Bauru, São Paulo, Brazil

Ida Maria Foschiani Dias Baptista, Marânia Cardoso Oelemann*, Diltor Vladimir Araújo Opromolla, Philip Noel Suffys*/+

Equipe Técnica de Microbiologia, Divisão de Pesquisa e Ensino, Instituto Lauro de Souza Lima, Bauru, SP, Brasil
*Laboratório de Biologia Molecular e Diagnóstico de Doenças Infecciosas, Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz-Fiocruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil

Little is known about transmission and drug resistance of tuberculosis (TB) in Bauru, State of São Paulo. The objective of this study was to evaluate risk factors for transmission of Mycobacterium tuberculosis strains in this area. Strains were collected from patients attended at ambulatory services in the region and susceptibility towards the main first line antibiotics was determined and fingerprinting performed. A total of 57 strains were submitted to susceptibility testing: 23 (42.6%) were resistant to at least one drug while 3 (13%) were resistant against both rifampicin and isoniazide. Resistant strains had been isolated from patients that had not (n = 13) or had (n = 9) previously been submitted to anti-TB treatment, demonstrating a preoccupying high level of primary resistance in the context of the study. All strains were submitted to IS6110 restriction fragment length polymorphism (IS6110-RFLP) and double repetitive element PCR (DRE-PCR). Using IS6110-RFLP, 26.3% of the strains were clustered and one cluster of 3 patients included 2 HIV-infected individuals that had been hospitalized together during 16 days; clustering of strains of patients from the hospital was however not higher than that of patients attended at health posts. According to DRE-PCR, 55.3% belonged to a cluster, confirming the larger discriminatory power of IS6110-RFLP when compared to DRE-PCR, that should therefore be used as a screening procedure only. No clinical, epidemiological or microbiological characteristics were associated with clustering so risk factors for transmission of TB could not be defined in the present study.

Key words: tuberculosis - transmission - drug resistance - fingerprinting - São Paulo - Brazil

Tuberculosis (TB) is still a calamity world wide and the main cause of death by a single infectious agent, namely Mycobacterium tuberculosis. The magnitude of the disease is associated with socio-economical level, spreading more easily in settings of agglomeration, mal nutrition and poverty, characteristics typical of third world nations (Gerhardt Filho & Hijjar 1993, Raviglione et al. 1997, Lima et al. 1997). Two recent developments have worsened the TB pandemic: the increase of multi-drug resistant bacilli resulting from inadequate therapies and indiscriminate use of antibiotics, and Aids, rendering individuals more susceptible to development of TB.

In Brazil, over 50 million people are probably infected by M. tuberculosis and although 83,309 new TB cases have been notified in 1997, the real number of new cases/year could be about 130,000. In the State of São Paulo, 18,266 new TB cases and an incidence of 55/100,000 were notified in 1995 (Brasil 1996). Few data exist on drug resistance in Brazil and general drug resistance in the State of São Paulo was reported to be 42.7% in 1992 (Silva et al. 1992); another survey determined primary resistance in that state during the nineties to be 16.5% and secondary resistance between 47.5% and 51.2% (Kritski et al. 1995).

The knowledge on the dynamics of TB transmission has recently improved thanks to the use of molecular typing techniques that allow the differentiation between M. tuberculosis strains. One of the most widely used genotyping techniques, restriction fragment length polymorphism (RFLP) based on the IS6110 insertion sequence (IS6110-RFLP), has been used to confirm TB outbreaks in hospitals and institutions for Aids patients, in asylums and prisons, to detect transmission of multi-drug resistant TB and to define risk factors associated with active transmission of TB in complex epidemiologic settings (Van Embden et al. 1993, Behr & Small 1997, Cohn & O'Brien 1998, Fandinho et al. 2000). A PCR-based fingerprinting procedure, the double repetitive element PCR (DRE-PCR), amplifying fragments localized between IS6110 and the repetitive polymorphic sequence rich in GC (PGRS) has recently been developed and demonstrated a discriminatory capacity similar of smaller than IS6110-RFLP (Friedman et al. 1995, Montoro et al. 1998).

In this study, we evaluated the level of drug resistance in M. tuberculosis strains isolated from patients attended in Health Units of the city of Bauru, compared IS6110-RFLP and DRE-PCR fingerprinting and verified possible nosocomial transmission of TB in a hospital.

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*Corresponding author: Fax: +55-21-2270.9997. E-mail: psuffys@ioc.fiocruz.br
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MATERIALS AND METHODS

Study setting and patient population - A study was performed on 57 strains of *M. tuberculosis* collected from different patients during the period of May 1996 to May 1999, including 42 patients attended at the Hospital Manoel de Abreu (HMA), 2 at the health care of the Instituto Lauro de Souza Lima and 13 at Health Units in the region of Bauru, the later isolated at the Instituto Adolfo Lutz.

The HMA is a general hospital that attends TB cases from the State of São Paulo; the patients arrolled in the present study were all from the Bauru region. During the study period, the hospital had 3 adjacent rooms with a total of 13 beds for infectious disease patients (including Aids), while a single room with 6 beds was available for patients with pulmonary disease; the distance between the rooms was 50 m.

Information on patient medical history and TB treatment were abstracted from medical charts and from a questionnaire containing additional demographic and epidemiologic data. The study was approved by the ethical committee of the Medical Faculty of Botucatu, São Paulo.

Susceptibility testing - All clinical samples except a lymph node puncture were of pulmonary origin and sent to the Laboratório de Micobacterias, Equipe Técnica de Microbiologia, Instituto Lauro de Souza Lima, for decontamination using the Petroff’s method, culture in Lowenstein-Jensen medium (L-J) and identification by evaluation of biochemical characteristics (David et al. 1994). The samples from the Instituto Adolfo Lutz we received as cultures. The susceptibility test was performed on Lowenstein-Jensen medium using the standard proportion method of Canetti et al. (1963). Briefly, resistance was defined when at least 1% of the number of colonies present on drug-free medium was observed on medium containing 0.2 mg/ml of isoniazid (INH), 40 mg/ml for rifampin (RMP), 200 mg/ml for pyrazinamide (PZA), 2 mg/ml for ethambutol (EMB) and 4 mg/ml for streptomycin (SM). Multi-drug resistance was defined as resistance to at least INH and RMP.

Genotyping by IS6110-RFLP and DRE-PCR - The *M. tuberculosis* strains were typed according to the standardized IS6110-RFLP method including internal marker in each sample as described by Van Embden et al. (1993). The RFLP profiles were analyzed and compared using GelCompar (Version 4.1; Applied Maths, Belgium), using the Dice coefficient of similarity and the UPGMA algorithm, and applying a 1.2% position tolerance. Only patterns with 100% similarity on the dendrogram that had been confirmed visually were considered as cluster. All strains were also submitted to DRE-PCR according to the methodology described in previous studies (Friedman et al. 1995, Montoro et al. 1998).

Statistical analysis - To verify whether there was a statistical significant association between strain clustering and any of the clinical, demographic, microbiological or epidemiologic data, the 2-squared test was used with 5% of significance.

RESULTS

Conventional analysis - The analysis of epidemiological and clinical characteristics of the 57 patients demonstrated that 34 patients had no previous history of TB of which 15 were HIV positive, 14 HIV negative and 5 had no HIV information. Previous TB had occurred in 19 patients, 10 HIV positive, 5 HIV negative and 4 unknown; among these, 8 patients had discharge for cure and 10 patients abandoned the treatment. In 4 patients, presence or absence of previous tuberculosis could not be determined. All strains were identified as belonging to the *M. tuberculosis* complex and drug sensitivity testing could be performed in 54 isolates; 3 contaminated prior to testing. Thirty-one (57.4%) of the strains were sensitive to all the drugs tested while 23 (42.6%) strains were resistant to at least one drug (Table I). Of the 23 samples resistant to drugs, 13 (56.5%) were isolated from patients with primary resistance and 8 (34.8%) from patients that had been previously treated; 2 (8.7%) strains were from patients with no information on previous treatment. Among the patients with resistant isolates, 14 (61%, including 6 with primary resistance, 6 with secondary resistance and 2 unknown) had been attended at the HMA, while 9 (39%, including 7 with primary resistance and 2 with secondary resistance) were from other Health Units; no statistically significant difference was observed between resistance levels at the hospital and other health units (p = 0.07; OR = 3.0) or between primary and secondary resistance at these sites (p = 0.37; OR = 0.29).

DNA fingerprinting analysis - Upon IS6110-RFLP analysis of the *M. tuberculosis* strains, IS6110 copy number varied from 3 to 17 and most had between 8 and 11 copies; only 1 strain had less than 6 IS6110 elements. A total of 46 different fingerprints were detected and 15 strains (26.3%) belonged to 5 different clusters (Figure). Cluster I

<table>
<thead>
<tr>
<th>TABLE I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Analysis of the resistance profile of the 23 Mycobacterium tuberculosis strains isolated from seropositive and seronegative patients in the region of Bauru, São Paulo, Brazil</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Patients</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistance to one drug</td>
<td>INH</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>PZA</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>SM</td>
<td>6</td>
</tr>
<tr>
<td>Resistance to two drugs</td>
<td>PZA, SM</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>INH, SM</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>INH, PZA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>INH, EMB</td>
<td>1</td>
</tr>
<tr>
<td>Resistance to three drugs</td>
<td>INH, SM, PZA</td>
<td>1</td>
</tr>
<tr>
<td>Multi-drug resistance</td>
<td>INH, RMP, PZA</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>INH, RMP, PZA, EMB, SM</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>100</td>
</tr>
</tbody>
</table>

INH: isoniazid, PZA: pyrazinamide, SM: streptomycin, RMP: rifampin, EMB: ethambutol
Cluster I (CI) comprised 3 strains from males of respectively 45, 29 and 30 years old, living in Bauru and having been hospitalized in HMA, including two HIV-infected patients that had been hospitalized in the same room together during 16 days. One was diagnosed for TB and submitted to hospitalization while TB was diagnosed in the other patient during the stay at the hospital. Both patients had positive BAAR but no information on initiation of treatment could be recovered from the clinical files. Cluster II (CII) contains strains from 3 HIV-positive males of 31, 34 and 32 years old, living in Bauru and hospitalized in the HMA during different periods. Cluster III (CIII) is formed by 4 strains including from 2 HIV negative females of 30 and 25 years, and 2 males of 22 and 25 years old with no information on HIV status; the former were hospitalized in the HMA during different periods but no information on hospitalization is available for the latter. Cluster IV (CIV) contains 2 strains from HIV-positive males of 22 and 33 years and without epidemiological link. The last cluster (CV) contains strains from 3 female patients of 21 (HIV-negative), 34 (HIV-positive) and 44 years old (HIV unknown); two of them were hospitalized in the HMA during the same period while there was no information on the other. Table II demonstrates that there was no statistically significant association between any clinical, epidemiological or bacteriological parameter and strain clustering.

All strains were also submitted to DRE-PCR: 32 different profiles with 0 to 7 bands were observed, while 31
strains belonged to 7 clusters. Cluster A contains 9 of the 12 strains with 1 band; cluster B and C contain respectively 4 and 3 of the 18 strains with 2 bands; cluster D contains 3 of the 15 strains with 3 bands; cluster E contains 3 of the 5 strains with 4 bands, cluster F contains all (4) strains with 5 bands and cluster G contains all (2) strains with 7 bands. The only strain that did not amplify neither did so after sample dilution. In order to evaluate reproducibility of the DRE-PCR, samples were submitted to two amplification reactions and in spite of the second one generated less PCR product, most of the strains demonstrated the same profile. Eleven strains demonstrated less bands after the second amplification but the position of the stronger bands was identical in all.

Upon comparing the two fingerprinting procedures, RFLP-IS6110 demonstrated a larger discriminatory capacity than DRE-PCR because strains with one or two band difference after RFLP-IS6110 had minor difference or identical profiles after DRE-PCR (Table III).

**DISCUSSION**

In Brazil, drug sensitivity testing of *M. tuberculosis* is not routinely performed but a small number of studies have shown a higher level of resistance in hospitals (Fandinho et al. 1999) when compared to the population attended in Health Units (Salem et al. 1990, Silva et al. 1992). In spite of São Paulo and Rio de Janeiro being the most troublesome regions in respect to TB, little is known about the behavior of the disease in satellite regions such as Bauru, a city with more than 350,000 inhabitants. During the last 6 years, TB incidence in Bauru was about 50/100,000 and one study performed at the HMA and at the Faculdade de Medicina de Botucatu reported resistance levels of 17.5% (Correia 1998). The rate of drug resistance of 42.6% observed in the present study is 2.5 times higher, demonstrating the need of routine sensitivity testing of *M. tuberculosis* strains in the region of Bauru. Contrary to what is usually reported on resistance in Brazil (Kritski et al. 1995, Fandinho et al. 1999), the rate of primary resistance was higher than that of secondary resistance and could either be explained by a high level of transmission of resistant strains or to the lack of correct information about previous history of TB. The lower cluster frequency observed in isolates from patients with primary resistance when compared to those obtained from patients with secondary resistance however strongly indicates that the
higher primary resistant level is not due to extensive trans-
mission or outbreaks of resistant strains. One study per-
formed by Monti et al. (1999) demonstrated a cure rate of
60% and treatment abandon of 14.1% in Bauru which could
be partly responsible for the high resistance rates in the
region.

The value of the IS6110-RFLP as an additional tool
for epidemiological studies of TB in Brazil has already been
demonstrated (Suffys et al. 2000) and both IS6110 copy
number and genetic polymorphism of the strains collected
in the present study were high, confirming our earlier data.
A clustering rate of 26.3% was observed, similar to the
rate observed in hospitals and in the general population
(Ivens de Araújo et al. 1998, Ferrazoli et al. 2000, Lourenço
et al. 2000). Based on the hypothesis that strains belong-
ing to a cluster are isolated from patients who are part of
a recent transmission chain (Gödfrey-Faussett 1999), in
Bauru, higher level of recent transmission was not asso-
ciated with sex, age, HIV status, previous TB, drug resis-
tance, type of resistance, previous treatment and type of
residence. Several studies have demonstrated that TB/
HIV co-infection and resistance to drugs are risk factors
for development of TB (Edlin et al. 1992, Coronado et al.
1993, Small et al. 1993, Ivens de Araújo et al. 1998), as was
also reported in a hospital in São Paulo (Ferrazoli et al.
2000) and in Rio de Janeiro (Fandinho et al. 2000). In
these studies however, a considerable part of the circulat-
ing strains has been analyzed; in the present study, only
one tenth of the 589 TB cases reported in the region of
Bauru during the 3-year period (Secretaria Municipal de
Saúde, pers. commun.) were submitted to strain typing. It
has been shown that sampling influences cluster fre-
quency (Glynn et al. 1999) and incomplete sampling is
probably one of the reasons for lack of detection of risk
factors for transmission. Nonetheless, combined analy-
sis of patient and fingerprinting data was highly sugges-
tive for transmission having occurred between two HIV
patients who had been hospitalized together. These pa-
tients lived in different neighborhoods and apparently
had no earlier social contact.

The largest cluster, CIII, was formed by strains with
an 8 bands profile that has recently been described in a
study that evaluated strains from of South and Southeast
Brazil and is also observed in other countries; this pattern
is characterizing a more easily spread strain or is more
stable (Suffys et al. 2000). Although it is generally as-
sumed that IS6110-RFLP clusters formed by patterns with
less than 6 IS6110 copies should be confirmed by a sec-
ondary typing procedure, recent studies demonstrate the
importance of confirmation also of certain high-copy
IS6110-RFLP clusters (Wall et al. 1999, Gillespie et al. 2000,
Niemann et al. 2000). In the present and a former study
(Suffys et al. 2000), strains belonging to the CIII cluster
had also identical DRE-PCR profile; in a recent finger-
printing study with strains from patients diagnosed at
health care services in Rio de Janeiro however, differences
were noted in DRE-PCR profiles obtained from strains with
this IS6110-RFLP pattern, demonstrating the need of bet-
ter characterization of these strains (Cardoso Oelemann
et al. unpublished observation).

Reports on comparison of DRE-PCR with other mo-
olecular typing techniques describe either a low discrimi-
natory power and poor reproducibility of the DRE-PCR
when compared to other techniques, including IS6110-
RFLP (Sola et al. 1998, Kremer et al. 1999) or similar dis-
mcrimatory power as RFLP (Montoro et al. 1998). Here,
the PCR procedure had less discriminatory power than
IS6110-RFLP, reflected by genetic polymorphism (respec-
tively 32 and 46 patterns), cluster frequency (respectively
55.3% and 26.3%) and concordance of both methodolo-
gies. Indeed, while all IS6110-RFLP clusters were con-
formed by DRE-PCR, clusters constructed by the latter
were larger and strains with one or two IS6110-
RFLP band difference sometimes belonged to a DRE-PCR
cluster. The differences observed among different PCR
reactions of the same strain were related to band intensity
and difficult interpretation of these low intensity bands
demands a rigorous control of the PCR conditions and
use of controls to evaluate reproducibility between reac-
tions.

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Padrão de Sensibilidade a Drogas das Cepas de Mycobac-

TABLE III
Comparison of the discriminatory power of IS6110-restriction
fragment length polymorphism (RFLP) and double repetitive
element-PCR (DRE)

<table>
<thead>
<tr>
<th>IS6110-RFLP Cluster/no. of strains/no. of bands</th>
<th>DRE-PCR Cluster/no. of strains/no. of bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>I/3</td>
<td>17</td>
</tr>
<tr>
<td>II/3</td>
<td>11</td>
</tr>
<tr>
<td>III/4</td>
<td>8</td>
</tr>
<tr>
<td>IV/2</td>
<td>11</td>
</tr>
<tr>
<td>V/3</td>
<td>9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<td>V/3</td>
<td>9</td>
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