Molecular analysis of *Mycobacterium tuberculosis* strains from an outpatient clinic in Porto Alegre, (RS)*

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Background: Tuberculosis is an ancient disease, which still remains one of the major ills faced by mankind in the 21st century. In recent decades, new technologies employing the knowledge gained from molecular biology studies have allowed for more accurate detection of tuberculosis and increased investigation of the etiology and epidemiology of the disease.

Aim: Evaluating the degree of similarity among strains of *Mycobacterium tuberculosis* provided by the Phthisiology Sector of Centro de Saúde Navegantes (Navegantes Health Clinic) in Porto Alegre, RS, Brazil.

Method: A retrospective study was performed involving RFLP typing of 55 sputum samples from outpatients examined at the Centro de Saúde Navegantes. The results of the genotyping were correlated to the conventional epidemiology data.

Results: A single pattern was seen in 39 (70.9%) of the isolates, whereas 16 isolates (29.1%) showed clustering patterns and were grouped into 8 clusters of 2 patients each. An epidemiological link was found for 6 (37.5%) of the 16 patients in the clusters.

Conclusion: The appropriate combination of conventional epidemiology and genotyping of *M. tuberculosis* contributes to a better understanding of the dynamics of tuberculosis transmission even when such a study is performed in a single, isolated health clinic.

Key words: *Mycobacterium tuberculosis*. Transmission. Polymorphism, Restriction Fragment Length. Epidemiology.

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INTRODUCTION

Tuberculosis (TB) has no flag, uniform or homeland. It has been a long-time companion of man, perhaps even since we became bipeds. Reports have shown evidence of TB in prehistoric human bones(1). Recent molecular studies show that the disease-causing agent, *Mycobacterium tuberculosis* (Mtb), has resided in the human organism for approximately 10,000 years, since the time at which humans first began to domesticate and herd animals(2). More than a hundred years after Robert Koch identified the disease-causing agent (in 1882), TB still has one of the highest rates of morbidity and mortality among all such chronic infections.

The increased incidence of TB over the last two decades has been attributed to the decline of public health systems, to the HIV pandemic and to the appearance of drug-resistant strains, as well as to the strong correlation between the disease and poverty(3).

In spite of having a nationwide TB control program, Brazil reports approximately 90,000 new cases of the disease every year, although it is estimated that the actual number is 129,000(4). In the state of Rio Grande do Sul, the rate of TB incidence in the year 2000 was 55.7/100,000 inhabitants, and the number of new cases reported was 6,152, 65% of which occurred in the metropolitan area of Porto Alegre(5).

Genotyping techniques used to differentiate among strains of Mtb have improved understanding of the dynamics of TB transmission. The active form of the disease develops as commonly after a recently acquired infection as it does after reactivation of an infection acquired long before(3,6). In a population-based study, it was postulated that patients with TB presenting single strains should be considered cases of TB by reactivation, and those presenting isolates sharing indistinguishable patterns be designated as belonging to clusters, representing the development of the disease from a recently acquired infection(6,7).

The restriction fragment length polymorphism (RFLP) technique uses the 6110 insertion sequence (IS*6110*) to analyze polymorphism. The RFLP is the genotyping method most widely used to differentiate among Mtb strains and has been useful in carrying out various types of epidemiologic studies(6,8).

Abbreviations used in this paper:

CSN– *Centro de Saúde Navegantes* (Navegantes Health Clinic)
EMB– Ethambutol
ETH– Ethionamide
HIV– Human immunodeficiency virus
INH– Isoniazid
IS– Insertion sequence
Mtb – *Mycobacterium tuberculosis*
PZA– Pyrazinamide
RMP– Rifampicin
RFLP– Restriction Fragment Length Polymorphism
TB– Tuberculosis

Because a better understanding of the dynamics of TB transmission is necessary, the present study aimed to assess the degree of similarity among strains of Mtb provided by the Phthisiology Sector of the *Centro de Saúde Navegantes* (CSN, Navegantes Health Clinic) in Porto Alegre. Molecular biology techniques were combined with conventional epidemiology.

METHODS

Patients and Samples

The Mtb isolates used in the study were provided by the Tuberculosis Laboratory of the IPB-LACEN/RS, which receives patient samples from the Phthisiology Sector of the Navegantes Health Clinic, an outpatient clinic in the northern part of Porto Alegre. We analyzed 55 sputum samples obtained from patients examined at the CSN between 1999 and 2000. Apart from primary culture, the samples were also tested for resistance to anti-TB drugs. Clinical and demographic data were obtained from patient files, as well as through review of laboratory records and data in the *Sistema de Informação de Agravos de Notificação* (SINAN, National Case Registry) database. The present study was approved by the Ethics Reasearch Committee of the *Fundação Estadual de Produção e Pesquisa em Saúde do Rio Grande do Sul* (Rio Grande do Sul State Foundation for Health Research).

Microorganism Culture and Susceptibility Test

The Kudoh & Kudoh method was used to cultivate clinic samples(9). Drug-susceptibility tests were performed according to the proportion method. As described by Canetti et al.(10), the following drugs were used: 40 μ g/mL of rifampin;

0.2 μ g/mL of isoniazid; 4 μ g/mL of streptomycin 2 μ g/mL of ethambutol; 100 μ g/mL of pyrazinamide; and 20 μ g/mL of ethionamide. Strains resistant to at least the combination of isoniazid and rifampin were considered multidrug-resistant.

The Restriction Fragment Length Polymorphism (RFLP)/IS6110 Technique

Genomic DNA was extracted using cetyltrimethylammonium bromide method and the IS6110 RFLP was carried out according to the protocol devised by Van Embden et al.(11).

Data Analysis

GelCompar software, version 4.2 (Applied Maths, Kortrijk, Belguim) and visual analysis were used to analyze digitized RFLP images. EpiInfo software, version 6.04d (Centers for Disease Control and Prevention, USA) was used to analyze the clinical and demographic data of patients.

RESULTS

Among the 55 Mtb isolates analyzed using the RFLP technique, 47 different band patterns were identified. Thirty-nine isolates (70.9%) presented a single pattern, whereas 16 isolates (29.1%) presented clustering patterns. Isolates having 6 or less copies of IS*6110* represented 5.4% of the total number of samples analyzed. The remaining 94.6% had 7 or more copies of the insertion element. The number of bands ranged from 2 to 8. The average and the trend were 10 copies of IS*6110* per isolate.

Isolates presenting 100% similarity were considered clusters. Out of the 55 isolates studied, 16 were grouped into 8 different clusters designated CL I, CL II, CL III, CL IV, CL V, CL VI, CL VII and CL VIII. Each cluster consisted of 2 isolates. Among the strain patterns identified through RFLP, three patterns belonged to the "A" family found in São Paulo(8) (Figure 1). Those patterns were visually compared with each other and then with the Mtb reference strain 14323, which was used as an external marker in each gel.

Cluster patients sharing the same household, living in the same neighborhood or belonging to the same family were considered to have an epidemiological link. Such an epidemiological link was observed for 6 patients (37.5%) in 3 of the 8 clusters identified. The links connecting those patients were based on residential proximity or family relation.

The average age was 42 among patients who developed TB after recent transmission and 43 among those who had reactivation TB. In addition, no significant gender-related age difference was found between cases resulting from recent transmission and those resulting from reactivation.

Between 1999 and 2000, the CSN reported 837 new TB cases. In 55 (6.57%) of those cases, samples were obtained and sent to the Tuberculosis Laboratory of the IPB-LACEN/RS. Only samples from patients not responding to Regime I were sent for culture, which explains the high proportion of previously treated patients (90.9%) in the study (Table 1).

Forty isolates (72.7%) had different resistance profiles and only 15 (27.3%) were sensitive to all drugs used in the treatment of TB. Twenty-three isolates (41.8%) were resistant to one or more drug and 17 (30.9%) were multidrug-resistant.

Concerning the factors that may be associated with the development of TB, no significant correlation was found between clinical/demographic data and *cluster* isolates (Table 1). Despite what has been mentioned, data obtained from patients infected with resistant strains show the significance of these patients as links in the chain of recent transmission.

DISCUSSION

Not so long ago, it was virtually impossible to identify individual Mtb strains. Therefore, tracking the transmission of a given strain in a region or institution was equally difficult. In recent years, genotyping techniques have been used to complement traditional methods of collecting epidemiological data.

Through RFLP analysis of Mtb isolates, 29.1% of patients evaluated in the present study were found to be in cluster, similar to proportions reported in studies involving isolates obtained from patients in Rio de Janeiro and São Paulo (22% and 23%, respectively)(12,8). In a study conducted in the metropolitan area of Porto Alegre, 34.5% of patients were found to be in cluster(13). Therefore, efforts aimed at control of the disease should focus not only on interrupting the chain of transmission but also on preventing reactivation TB. Our results

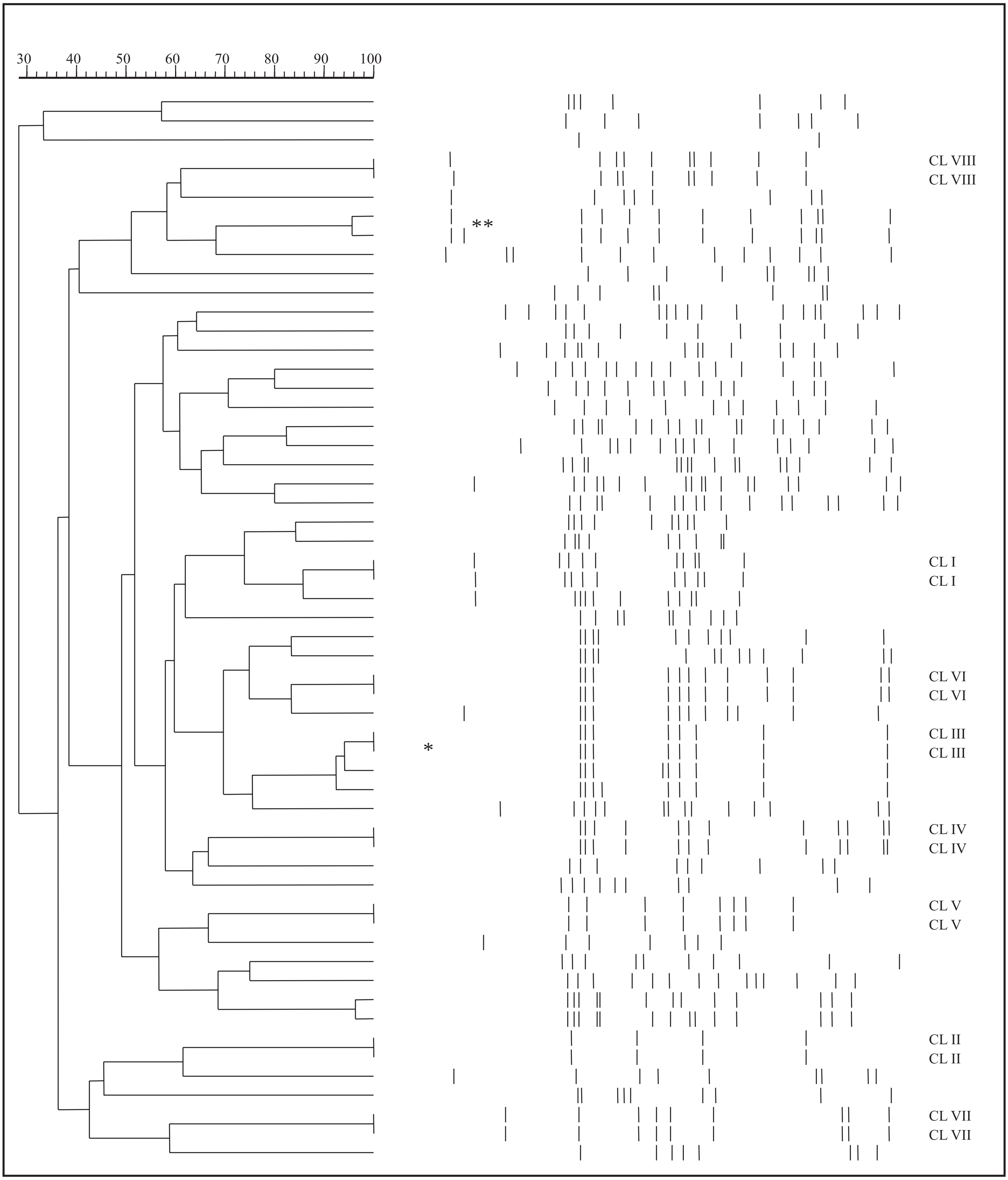

Figure 1. Dendrogram representing the various RFLP patterns found in *Mycobacterium tuberculosis* isolates obtained from patients treated at the *Centro de Saúde Navegantes*. Clusters were designated CL I, CL II, CL III, CL IV, CL V, CL VI, CL VII, and CL VIII. *Patterns of strains beloging to the "A" family found in São Paulo[(8)] ***M. tuberculosis* reference strain 14323

TABLE 1
Clínical and demographic characteristics of the patients evaluated

Characteristic	Cluster *n* (%)	Non-cluster *n* (%)	*p* value	Total
Gender				
Male	13 (28.9)	32 (71.1)	0.6	45 (81.8)
Female	3 (30)	7 (70)		10 (18.2)
Race				
Caucasian	13 (30.2)	30 (69.8)		43 (78)
Black	2 (33.3)	4 (66.7)	0.77	6 (11)
Unknown	1 (16.7)	5 (83.3)		6 (11)
Previous treatment				
No	3 (60)	2 (40)	0.14	5 (9.1)
Yes	13 (26)	37 (74)		50 (90.9)
Resistance				
No	2 (13.3)	13 (86.7)	0.1	15 (27.3)
Yes	14 (35)	26 (65)		40 (72.7)
Multidrug resistance				
No	11 (28.9)	27 (71.1)	0.6	38 (69.1)
Yes	5 (29.4)	12 (70.6)		17 (30.9)
HIV				
Negative	9 (34.6)	17 (65.4)		26 (47.3)
Positive	1 (14.3)	6 (85.7)	0.56	7 (12.7)

are very similar to those of other studies carried out in Brazil (Table 2). As described in various other studies(14-16), patterns with an average of 10 bands per isolate were observed.

Even though 81.8% of patients were male, the rate of recent transmission among males did not differ from that found for female patients. This indicates that, although male gender may be a risk factor for the development of TB, it is not necessarily correlated with higher rates of recent transmission(6,8,17,18). In addition, no significant race-related difference was found in the percentage of cluster patients, although there is a greater tendency among black patients for developing the disease from recent transmission. In the present study, the small number of black patients involved probably accounted for the lack of any significant difference, since blacks represent only 5.2% of the total Rio Grande do Sul population, whereas whites represent 86.3%(19). It has been shown that blacks are more likely to develop the disease from recent transmission(6). Although this is probably related to socioeconomic status, there have been studies investigating the possibility that genetic factors influence susceptibility(20).

In the present study, 14.3% of HIV-positive patients developed TB from recent transmission, which was not significantly related the proportion of HIV-negative patients (34.6%) who developed the disease under the same conditions . Probably due to the small sample size, our data differ from what has been reported in the literature, in which HIV-positive patients have been shown to be more likely to develop TB – both from reactivation and from recently acquired infections(3).

Through analysis of the clinical data, an epidemiological link was identified for 6 patients (37.5%) who developed the disease from recent transmission. The percentage found in the present study was similar to that found in studies performed in Hong Kong (30.2%) and in the USA (Missouri: 46%; Arkansas: 42%)(21,22,23).

The high level of resistance to at least one drug (72.7%) observed in the present study is directly related to sample selection. A history of previous treatment is a predisposing factor for resistant bacilli selection(24), as evidenced by the fact that 90.9% of the patients evaluated in the present study had previously undergone treatment.

TABLE 2
Comparison with results from other molecular epidemiological studies conducted in Brazil

CopiesOrigin of isolates	Locale	Period	*n*	of IS*6110*	Cluster pattern	HIV status	Reference
Porto Alegre, RS	Outpatient clinic	1999-2000	55	2-18	29.1%	7 positive 26 negative 22 unknown	Presente Estudo
RJ	Hospital	1993-1994	19	6-15	53%	15 positive 4 negative	Ivens-de-Araújo et al.(27)
RJ	Hospital	1990-1994	32	———	25%	———	Lourenço et al.(28)
Bauru, SP	Outpatient clinic	1996-1999	57	3-17	26.3%	25 positive 19 negative 13 unknown	Baptista et al.(29)
Campinas, SP	Hospital	1996-1999	78	6-21	22.3%	———	Calusni et al.(16)
RJ	Hospital	1993-1994	120	3-18	19%	30 positive 67 negative 23 unknown	Fandinho et al. (15)
SP	Outpatient	1995-1997	293	2-20	33%	151 positive 142 negative	Ferrazoli et al.(8)clinic
RJ, RS	Outpatient Clinic Hospital	1991-1994	219	1-18	22%	———	Suffys et al.(12)

The correlation between recent transmission and resistance was not significant, although 35% of patients presenting resistant strains developed TB after a recent transmission. Other studies have also shown that patients with resistant strains contribute significantly to cluster formation(6,8,15,25,29), which could be related to the difficulties encountered in detecting resistance and in treating these patients. In comparison to patients infected with sensitive strains, patients with drug-resistant strains require treatment that is more protracted, toxic and expensive, present lower cure rates, and tend to remain infected for longer periods(26).

Even though our study was limited to patients treated at the CSN, considerable diversity was seen among Mtb strain patterns, and a high rate of epidemiologic links was found among patients belonging to clusters. This can be attributed to the appropriate combination of conventional epidemiology techniques and genotyping, which provided an overview of the transmission dynamics of the disease at this location.

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