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ERIKA NODA NOGUTI

Genotyping of *Mycobacterium tuberculosis* isolates from low endemic setting in south of Brazil

Maringá
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Dissertação apresentada ao Programa de Pós-Graduação em Biociências Aplicadas à Farmácia do Departamento de Análises Clínicas, Centro de Ciências da Saúde da Universidade Estadual de Maringá, como requisito parcial para obtenção do título de Mestre em Biociências Aplicadas à Farmácia
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Genotipagem de *Mycobacterium tuberculosis* isolados de uma região de baixa endemicidade no sul do Brasil

RESUMO

A tuberculose (TB) é um importante problema de saúde pública mundial, sendo a principal causa de mortes devido a um agente infeccioso. O objetivo desse trabalho foi fornecer informação sobre a diversidade genética de *Mycobacterium tuberculosis,* isolados de pacientes com TB pulmonar atendidos no Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC), utilizando as metodologias *Spoligotyping* e MIRU-VNTR. Os resultados foram relatados no artigo "Genotyping of *Mycobacterium tuberculosis* isolates from low endemic setting in south of Brazil". Com os perfis de *Spoligotyping* e MIRU-VNTR dos isolados de *M. tuberculosis* foram construídos dendrogramas para avaliar o grau de relação entre os isolados. A partir desta avaliação pode-se verificar que a TB em nossa região desenvolve-se predominantemente devido à reativação endógena de uma infecção latente. Foi realizada também uma comparação da capacidade discriminatória entre as metodologias utilizadas (*Spoligotyping* e MIRU-VNTR), obtendo-se melhor capacidade discriminatória dos isolados combinando as duas metodologias.

Palavras-chave: Tuberculose; *Mycobacterium tuberculosis*; Epidemiologia Molecular; *Spoligotyping*; MIRU

Genotyping of *Mycobacterium tuberculosis* isolates from low endemic setting in south of Brazil

ABSTRACT

Tuberculosis (TB) is an important public health problem worldwide and the leading cause of deaths by an infectious agent. The objective of this study was provide information about genetic diversity of *Mycobacterium tuberculosis*, isolated from patients with pulmonary TB attended at the Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC), using Spoligotyping and MIRU-VNTR methodologies. The results were reported in the paper "Genotyping of *Mycobacterium tuberculosis* isolates from low endemic setting in south of Brazil". From the Spoligotyping and MIRU-VNTR patterns of *M. tuberculosis* isolates dendrograms were constructed to evaluate the relationship of the isolates. The results show that TB in our region predominantly develops from endogenous reactivation of a latent infection. It was also carried out a comparison of discriminatory power between the methodologies used (Spoligotyping and MIRU-VNTR), obtaining better discriminatory capacity of the isolates combining the two methodologies.

Keywords: Tuberculosis; *Mycobacterium tuberculosis*; Molecular Epidemiology; Spoligotyping; MIRU

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SUMÁRIO

CAPÍTULO I

FUNDAMENTAÇÃO TEÓRICA

O gênero *Mycobacterium* é o único representante da família *Mycobacteriaceae* e pertence à sub-ordem *Corynebacteraceace* e ordem A*ctinomycetales*. Bactérias desse gênero possuem algumas características taxonômicas comuns, como álcool-ácido resistência quando exposto a coloração tintorial Ziehl-Neelsen e DNA com alto teor em guanina e citosina[(1)]. Atualmente, já foram identificadas mais de 120 espécies desse gênero, e poucas foram reportadas como patogênicas em humanos e/ou animais[(2)].

A tuberculose (TB) ou Peste Branca é uma doença infecto-contagiosa causada por bacilos pertencentes ao complexo *Mycobacterium tuberculosis* (MTC). O complexo engloba micobactérias que apresentam genoma extremamente similar, próximo a 100%[(3)] e é composto pelo *Mycobacterium tuberculosis*, o principal agente da TB em humanos; *M. bovis*; *M. bovis* Bacille Calmette-Guérin (BCG), cepa atenuada utilizada na vacinação; *M. africanum*; *M. microti*; *M. canettii*[(4)]; *M. caprae*[(5)]; e *M. pinnipedii* [(6)].

A TB acomete principalmente pessoas na faixa etária correspondente a plena capacidade produtiva, afetando os setores de mais baixa renda da população, vindo a acarretar enorme prejuízo econômico ao país[(8)].

Estima-se que um terço da população mundial esteja infectada com o bacilo *M. tuberculosis*. Essa infecção pulmonar ocorre pela inalação de gotículas contendo os bacilos expelidos por um indivíduo com TB na forma ativa[(9)]. Na minoria dos indivíduos infectados (5-10%) ocorre o desenvolvimento da doença dentro de 2-5 anos após a infecção (TB primária). No restante dos indivíduos (90-95%), essa infecção permanece em estado latente (TB latente), onde o bacilo persiste no indivíduo no estado dormente. Entretanto, estima-se

que ocorra reativação do bacilo em 10% dos indivíduos com TB latente, ocasionando a TB secundária, devido a uma falha da vigilância do sistema imune[10].

Tosse e expectoração persistente por mais de três semanas são os principais sintomas da TB na forma ativa. Outras manifestações podem ser perda de peso, febre, sudorese noturna, cansaço físico e dores torácicas[9].

A epidemia da infecção pelo vírus HIV tem mudado radicalmente a epidemiologia da TB, uma vez que HIV e TB estão intimamente associados. Enquanto HIV promove a progressão da TB latente para doença ativa, a TB é uma das principais infecções que matam pessoas com HIV[11]. Nos pacientes com HIV/AIDS o início rápido da terapia anti-retroviral em indivíduos com TB latente poderia diminuir a incidência de TB nesses indivíduos[12].

O diagnóstico da TB deve basear-se, além da avaliação clínica, na bacteriologia, radiologia, prova tuberculínica e histopatologia (utilizado principalmente na investigação de formas extrapulmonares)[13].

A pesquisa bacteriológica é um método de importância fundamental, tanto para diagnóstico como para controle de tratamento e é realizada pela pesquisa direta (baciloscopia) e cultura de Bacilos Álcool-Ácido Resistente (BAAR). Por ser de execução rápida, fácil e de baixo custo, a baciloscopia favorece uma ampla cobertura diagnóstica, identificando a principal fonte de infecção (doentes bacilíferos). A cultura é indicada para os suspeitos de TB pulmonar persistentemente negativo ao exame direto, para o diagnóstico de formas extrapulmonares e nos casos de suspeita de resistência bacteriana às drogas, seguida do teste de sensibilidade. O exame radiológico é auxiliar no diagnóstico da TB, permitindo a seleção de portadores de imagens sugestivas de TB e de outras patologias. A prova tuberculínica (PPD) é outro teste auxiliar no diagnóstico, no entanto uma prova positiva, isoladamente, indica apenas infecção por BAAR e não é suficiente para o diagnóstico da TB doença[13].

TB é uma doença curável, desde que obedecidos os princípios da moderna quimioterapia. O tratamento de casos novos tanto para a forma pulmonar quanto extrapulmonar adotado atualmente no Brasil é composto pelos antibióticos Rifampicina (R), Isoniazida (H) e Pirazinamida (Z) com duração de seis meses, sendo os dois primeiros meses com R, H e Z e os outros quatro, com R e H[13].

Como conseqüência da falha na infraestrutura de saúde pública cepas multidroga-resistente (MDR-TB) e extensivamente droga-resistente (XDR-TB) emergiram. De acordo com normas internacionais cepas MDR-TB possuem resistência a pelo menos duas das principais drogas anti-tuberculosas, H e R e requer tratamento prolongado com quimioterápicos caros de segunda linha que são tóxicos[14]. E cepas XDR-TB possuem além da resistência a H e R, resistência a um fármaco injetável de segunda linha (capreomicina, canamicina ou amicacina) e a uma fluoroquinolona[15].

Uma das formas de prevenir a TB é a vacinação com a BCG (Bacille Calmette-Guérin), derivada da cepa atenuada de *Mycobacterium bovis.* A vacina BCG exerce notável efeito protetor contra as manifestações graves da infecção primária, como as disseminações hematogênicas e a meningoencefalite, mas não evita a infecção pelo *M. tuberculosis*[13].

O Brasil ocupa o 16º lugar entre os 22 países responsáveis por 80% do total de casos de TB no mundo. Estima-se que, em 2006, ocorreram cerca de 94.000 casos novos, resultando numa incidência de 50/100.000 habitantes e 7.600 óbitos[16]. No estado do Paraná, sul do Brasil, a taxa média de incidência da TB é mais baixa que a média nacional ficando na casa de 27,52/100.000 habitantes[17].

Atualmente, ferramentas moleculares são utilizadas no estudo da dinâmica de transmissão de doenças infecciosas. São várias as aplicações dessas técnicas moleculares no estudo da epidemiologia da TB, como discriminação entre TB recorrente devido à reinfecção exógena ou reativação, determinação da expansão geográfica de cepas, monitoramento da

transmissão de cepas resistentes aos fármacos antituberculose, determinação da freqüência de resistência a fármacos em diferentes grupos, detecção de infecções mistas nos pacientes, avaliação dos programas de controle, identificação de transmissão cepa-específica, identificação de tipos de cepas predominantes (cepa clonal) em estudos populacionais, identificação de cepas hipervirulentas em populações, investigações da evolução do *M. tuberculosis*[18], avaliação de contaminação laboratorial cruzada[19], entre outros.

O conhecimento sobre a dinâmica da transmissão da TB tem sido melhorado desde a introdução de técnicas de DNA *fingerprinting* de *M. tuberculosis* na década de 90, quando uma variedade de marcadores genéticos foram identificados[20].

IS*6110* é um elemento de inserção específico presente no genoma do MTC, embora possa estar ausente em alguns membros desse complexo[21]. Usualmente *M. tuberculosis* têm de 8-18 cópias de IS*6110*, porém este número pode variar de 0-25[22]. Em 1993, *IS*6110 *Restrition Fragment Lenght Polymorphism* (RFLP) foi adotado como método padrão ouro para tipagem de isolados de *M. tuberculosis*[23]. Este método se baseia na digestão da fita de DNA micobacteriano pela enzima de restrição *Pvu*II, que gera fragmentos de diferentes comprimentos de pares de base. Os fragmentos são então separados em gel de agarose e, após *Southern Blotting*, hibridizados por sonda de DNA direcionados para o IS*6110*[24].

Entretanto, apesar de ser 100% reprodutível, ter alto grau de discriminação e especificidade[20], o método *IS*6110-RFLP é trabalhoso, requer semanas para cultura dos isolados para obter altas concentrações de DNA, sofre problemas de interpretação e portabilidade do complexo padrão de bandas e tem baixo poder discriminatório entre isolados com baixo número de cópias (<6) de IS*6110* [25].

Spoligotyping (*Spacer Oligotyping*) é o segundo método de tipagem molecular mais utilizado em estudos epidemiológicos da TB após IS*6110*-RFLP[21]. Este método se baseia na análise da presença ou ausência de 43 espaçadores no *locus* DR (*direct repeats*), um *locus*

altamente polimórfico no genoma do bacilo pertencente ao MTC[26]. O *locus* DR contém múltiplas e conservadas repetições diretas de tamanho de 36pb e intercaladas com seqüências espaçadoras não repetitivas de 35-41pb de comprimento[27]. Inicialmente uma amplificação pela *Polymerase Chain Reaction* (PCR) do *locus* DR é realizada e após, os produtos são hibridizados com 43 oligunucleotídeos complementares às regiões espaçadoras variáveis localizadas entre as DRs que estão imobilizados em uma membrana de Nylon (Biodyne C)[28]. *Spoligotyping* é um método simples e rápido, porém com poder discriminatório menor do que o do IS*6110*-RFLP, quando isolados com alto número de cópias da IS*6110* são analisados. No entanto, seu poder discriminatório é maior para a avaliação de cepas com baixo número de cópias da IS*6110*[29].

Spoligotyping tem a vantagem dos resultados poderem ser descritos por método padronizado na forma de códigos, o que facilita a comparação entre diferentes laboratórios[30] e de ser encontrado um banco de dados internacional de *Spoligotyping*, o SpolDB4, que atualmente contém 1939 perfis de *Spoligotypes* compartilhados (*Shared Types* – STs) representando um total de 39.609 isolados de 121 países (www.pasteur-guadeloupe.fr:8081/SITVITDemo/).

Outro método utilizado em estudos epidemiológicos da TB é o MIRU - *Mycobacterial Interspersed Repetitive Units*. Neste método são analisadas seqüências homólogas de 46-100pb de DNA que se repetem sequencialmente (*tandem repeats)* e que estão dispersas em regiões intergênicas no genoma de *M. tuberculosis*. Foram identificados 41 *loci* contendo MIRU, dos quais 12 demonstraram-se polimórficos no número de cópias dessas unidades repetitivas sendo, portanto qualificadas como *Variable Number of Tandem Repeats* – VNTR. MIRU-VNTR é um método baseado na realização da PCR utilizando *primers* específicos para cada *locus* seguido de eletroforese em gel de agarose para análise dos tamanhos dos fragmentos de DNA amplificados. Os tamanhos dos *amplicons* são estimados pela

comparação com pesos moleculares padrões para determinação do número de *tandem repeats* presentes em cada *locus*[31].

A utilização desses 12 *loci* MIRU-VNTR associada ao spoligotyping apresentou discriminação similar ao RFLP-IS*6110* na maioria dos isolados analisados por Cowan e colaboradores (2005), mas ainda sendo necessária a utilização do RFLP para os isolados agrupados quando for necessária discriminação adicional[32].

Recentemente foi definido um grupo de 24 *loci* MIRU-VNTR, incluindo um subgrupo de 15 *loci* altamente discriminatório para ser utilizado como ferramenta de primeira linha na tipagem molecular de isolados de *M. tuberculosis*[33]. Estudos com esses 15 ou 24 *loci* MIRU-VNTR apresentaram poder discriminatório comparados aos do IS*6110*-RFLP, principalmente quando combinado com *Spoligotyping*[34].

JUSTIFICATIVA

Sistemas que diferenciam isolados de *M. tuberculosis* epidemiologicamente relacionados, de outros não relacionados, são ferramentas poderosas numa investigação de surtos de infecção hospitalar, ou comunitária, bem como para diferenciar reativação endógena de uma re-infecção exógena no caso da tuberculose. A utilização de metodologias moleculares de fácil execução, custo acessível e principalmente baseadas na PCR tem apresentado considerados avanços nas genotipagem que permitem a diferenciação de isolados de *M. tuberculosis* permitindo a determinação do grau de relação entre esses isolados de diferentes pacientes participantes no presente estudo. Este trabalho se fez necessário considerando que existem poucos estudos sobre a variabilidade genética das linhagens circulantes de *M. tuberculosis* no Brasil e na 15° Regional de Saúde de Maringá, estado do Paraná este é o primeiro estudo em que temos conhecimento.

As informações obtidas em formato binário servirão para construir um banco de dados dos isolados obtidos e comparar, os perfis prevalentes em nossa região com os perfis de *Spoligotyping* e MIRU-VNTR em outras regiões do Brasil e outros países.

OBJETIVOS

GERAL

Caracterizar molecularmente amostras de *M. tuberculosis* isoladas de pacientes com tuberculose pulmonar atendidos no Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC) que é laboratório de referência da 15ª Regional de Saúde de Maringá, Paraná.

ESPECÍFICOS

Analisar e interpretar os perfis obtidos utilizando as técnicas *Spoligotyping* e MIRU-VNTR com os isolados de *M. tuberculosis*.

Avaliar o grau de relação entre os isolados de *M. tuberculosis* obtidos de pacientes com tuberculose.

Comparar os perfis de S*poligotyping* e MIRU-VNTR encontrados com os perfis do isolados de outras regiões e em outros países.

Comparar a capacidade discriminatória das técnicas *Spoligotyping* e MIRU-VNTR.

Verificar a principal forma de ocorrência da tuberculose em nossa região, por transmissão recente ou por reativação endógena de uma tuberculose latente.

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CAPÍTULO II

Artigo: "GENOTYPING OF *Mycobacterium tuberculosis* ISOLATES FROM LOW ENDEMIC SETTING IN SOUTH OF BRAZIL."

GENOTYPING OF *Mycobacterium tuberculosis* ISOLATES FROM LOW ENDEMIC SETTING IN SOUTH OF BRAZIL

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Abstract

Objective: To provide information about the genetic diversity and prevalent genotype of *Mycobacterium tuberculosis* and compare the usefulness of two methodologies in epidemiological study of tuberculosis in low endemic setting in the south of Brazil.

Methods: We employed spoligotyping and MIRU-VNTR technique to genotype *M. tuberculosis* isolates from patients with pulmonary tuberculosis.

Results: The 93 isolates analyzed by spoligotyping were divided into 36 different patterns and 30 were described in the SITVIT database. Latin American and Mediterranean, Haarlem and T family were responsible for 26.9%, 17.2% and 11.8%, of tuberculosis cases respectively. From the 84 isolates analyzed by MIRU-VNTR, 58 showed unique pattern and 26 belonged to 9 clusters. The MIRU loci 40, 23, 10 and 16 were the most discriminatory. MIRU-VNTR and spoligotyping combined showed 85.7% of discriminatory power (Hunter-Gaston Index, HGI=0.995).

Conclusion: Spoligotyping and MIRU-VNTR typing combined are useful tools for epidemiological study in this low endemic setting in the south of Brazil and tuberculosis probably predominantly develops through reactivation of latent infection.

Keywords: *Mycobacterium tuberculosis*; Tuberculosis; Molecular Epidemiology; Spoligotyping; MIRU

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Introduction

Tuberculosis (TB) is an important public health problem worldwide, with an estimated 9.27 million new cases in 2007, and the leading cause of deaths by an infectious agent. In Brazil, the estimated incidence rate is 48/100 000 inhabitants.[1] Brazil has five distinct geographic regions, among which the distribution of TB cases varies greatly. In Paraná State, south of Brazil, the average rate of tuberculosis is 27.52/100 000 inhabitants.[2] Maringá and the small cities surrounding it form a low endemic area of TB in Paraná State with an incidence rate of 22.62/100 000 in 2007 (personal communication).

Genotyping methods have been extensively applied in TB epidemiological study worldwide.[3,4,5] Those studies are based on the assumption that patients with genotypically clustered strains are epidemiologically linked. It represents recent transmissions, while those infected with different types of strains are not. Genetic markers should be sufficiently polymorphic to distinguish unrelated strains and stable enough to identify isolates of the same strains. Therefore, the typing methods must be reproducible, discriminatory and easy to perform.

Some PCR-based techniques are being now used to differentiate *Mycobacterium tuberculosis* isolates. Nowadays, genotyping approaches targeting the variable number of tandem repeats (VNTR) analysis based on mycobacterial interspersed repetitive units (MIRU) is the most promising. This technique is based on the variability found at 12 specific loci interspersed throughout the mycobacterial genome.[6] Recently a 15 or 24 loci MIRU-VNTR genotyping[7,8,9] have been evaluated and applied for molecular epidemiological typing in mycobacteria.

Spoligotyping is the second most widely used method after IS*6110* based fingerprinting[10] and it is based on the presence or absence of a set of target sequences in the direct repeat (DR) locus in *M. tuberculosis* complex genome.[11]

Spoligotyping combined with MIRU have been used to replace the restriction fragment length polymorphism (RFLP) typing based on the insertion sequence IS*6110*, the "gold standard" for genotyping *M. tuberculosis* since 1993, an expensive, laborious and lengthy methodology that requires weeks of *M. tuberculosis* culturing, specific software to analyze the RFLP band-patterns making it difficult to interpret and exchange data. In addition to that, this method is limited for genotyping *M. tuberculosis* isolates containing low number of IS*6110* copies.

The aim of our study was to provide initial information about the genetic diversity and prevalent genotype of *M. tuberculosis* isolates in a low endemic setting in the south of Brazil based on spoligotyping and MIRU-VNTR typing and to compare the usefulness of these methodologies in epidemiological study of TB in that area.

Materials and methods

Study isolates

A total of 93 *M. tuberculosis* isolates analyzed in this study were obtained from the culture collections of the Clinical Bacteriology Laboratory, Department of Clinical Analysis of State University of Maringá, Paraná State, south of Brazil, a reference TB laboratory that attends patients from Maringá and other cities in the northwest of Paraná State, Brazil. The culture collection was obtained from a sample bank containing isolates originally cultured from November 2005 to June 2008. The isolates were cultured in Difco™ Lowenstein Medium Base (Becton, Dickinson and Company, Sparks, MD, USA) and identified as *M. tuberculosis* by the conventional biochemical tests[12] and molecular biology.[13] The following retrospective demographic and epidemiological data were collected for all patients by review of a national TB notification database (SINAN – National Diseases Notification System): city and zip code

of residence at the time of diagnosis, age, sex, ethnicity, HIV status, sample susceptibility profile, alcoholism and the occurrence of other diseases.

DNA extraction

DNA from *M. tuberculosis* was extracted from a subculture on Difco™Lowenstein Medium Base, as described by Gonzalez-y-Merchand et al.[14] with minor modifications. Briefly, a loopful of bacterial growth was suspended in 6M guanidine hydrochloride (Sigma Chemical Co., St. Louis, Mo, USA) and bacilli were lysed by freezing at -20°C for 30 min and followed by heating at 65ºC for 10 min. This procedure was repeated twice. DNA was further extracted by 2 volumes of phenol-chloroform-isoamyl alcohol (25:24:1, v/v), followed by two steps of extractions with chloroform-isoamyl alcohol (24:1, v/v). DNA was purified by ethanol precipitation, dissolved in 50 µl of Tris-EDTA, pH 8.0 (TE buffer) and stored at –20ºC until the use. DNA concentration was determined by ultraviolet spectrophotometry.

Spoligotyping

Spoligotyping was performed in all the 93 *M. tuberculosis* isolates to detect presence or absence of 43 spacers by using the standard method.[15] Briefly, DR region was amplified using 1 µl of mycobacterial DNA in 24 µl of a reaction mixture containing 0.4 µM of each primers DRa 5'-GGTTTTGGGTCTGACGAC-3' (biotinylated 5' end) and DRb 5'-CCGAGAGGGGACGGAAAC-3', (Integrated DNA Technologies, Inc. Coralville, USA) and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA) according to manufacture's instructions. The amplification of the DNA was carried out in a TC-512 thermal cycler (Techne, UK). PCR products were hybridized with a set of 43 spacer oligonucleotides covalently linked to the spoligo-membrane (Isogen Life Sciences, The Netherlands), according to the manufacturer's instructions. Bounded fragments were detected

by chemiluminescence after being incubated with streptavidin-peroxidase conjugate (Boehringer, Ingelheim, Germany) and assessed by an enhanced chemiluminescence system (ECL; GE Healthcare UK Limited, Buckinghamshire, UK). Spoligotypes were reported by using an octal code where the 43-digit binary representing the 43 spacers ("1" is hybridization and "0" is no hybridization) was divided into 14 sets of three digits (spacers 1 to 42) plus one additional digit (spacer 43). Each three digits set was converted to octal code (000 = 0, 001 = 1, 010 = 2, 011 = 3, 100 = 4, 101 = 5, 110 = 6 and 111 = 7) with the final digit remaining either 1 or 0 yielding a 15-digit octal designation.[16]

MIRU-VNTR typing

MIRU-VNTR typing was performed only in 84 out of 93 *M. tuberculosis* isolates at Laboratory of Mycobacteria Dr. Hugo David in the School of Pharmaceutical Science, São Paulo State University, Araraquara, São Paulo, Brazil. The isolates were genotyped by PCR amplification of the original 12 MIRU-VNTR loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, and 40) as described by Supply et al.[6] and Mazars et al.[17] in a PTC-100 thermal cycler (MJ Research**,** Ramsey, Minnesota, USA**).** Each locus was amplified individually with 2 µl of mycobacterial DNA in 23 µl of a reaction mixture containing 0.4 µM of loci respective primers and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA) according to manufacture's instructions. The PCR conditions for each set of primers were described elsewhere.[18] The PCR amplicon was subjected to electrophoresis in 2.0% w/v agarose gel (Invitrogen Life Technologies, São Paulo, Brazil). The 50 and 100-bp DNA Ladders (Invitrogen Life Technologies, São Paulo, Brazil) were used as molecular markers. The gels were stained with ethidium bromide and visualized under ultraviolet light and photodocumented with Alpha-imager 2200 (Alpha Innotech Corporation**,** San Leandro, CA, USA**).** The size of PCR fragment was determined by visual comparison with the molecular

markers and the MIRU allele scoring was determined according to Mazars et al.[17] and Supply et al.[19] The results from each of the 12 loci were combined to create 12-digit allelic profiles.

Interpretation of genotyping results

The found spoligotypes were compared to international database SITVIT, which is an updated version of the published SpolDB4.0 database,[20] and it is available at www.pasteur-guadeloupe.fr:8081/SITVITDemo/. BioNumerics software (version 4.45; Applied Maths, Sint-Martens-Latem, Belgium) was used for analysis of spoligotyping and MIRU-VNTR patterns. Dendrograms were constructed for spoligotyping, MIRU-VNTR and these methodologies combined. The genetic distance was built employing the UPGMA algorithm (Unweighted Pair Group Method with Arithmetic Mean).[21] The evaluation of the discriminative power of each typing method separately as well as in combination was undertaken by using the Hunter-Gaston index (HGI),[22] which is based on the probability that two unrelated strains sampled from the population test will be placed into different typing groups. Allelic diversity of each locus MIRU-VNTR was classified as "highly discriminant" (HGI > 0.6), "moderately discriminant" ($0.3 \leq HGI \leq 0.6$) and "poorly discriminant" (HGI < 0.3).[23]

Results

The study population came from 8 cities (Maringá and other 7 small neighboring cities) and it was consisted of 78 males (83.9%) and 15 females (16.1%). The age of the patients ranged from 14 to 83 years old (mean age was 40.0 years). Fifty two (55.9%) patients were white, 6 (6.4%) black, 22 (23.7%) mixed and 13 (14.0%) were unknown. The results of the HIV testing were available for 43 (46.2%) patients. Among these, 4 (9.3%) were tested positive. The use of alcohol was known in 58 patients, where 23 (39.7%) patients reported to use it.

The presence of other diseases such as diabetes and mental illness was known in 55 patients, where 3 (5.5%) patients reported to have diabetes and 2 (3.6%) mental illness. The tests for drug resistance were performed in 69 (74.2%) isolates. Resistance was detected in 3 clinical isolates (4.3%), one was resistant to rifampicin and 2 were multidrug-resistant (MDR-TB).

Spoligotyping

Thirty six different spoligotyping patterns were observed in the 93 *M. tuberculosis* isolates. Spoligopatterns of 83 isolates (89.2%) were classified according to SITVIT database, which 65 isolates were clustered into 12 Shared International Types (ST), comprising from 3 to 13 isolates each cluster and the remaining 18 isolates showed unique ST. The main STs found in the present study were ST46 (n=13, 15.7%), ST20, ST42 and ST47 with 6 isolates (7.2%) each one.

Ten isolates (10.8%) have not been identified in the SITVIT database yet, being 4 orphans (40.0%) and the remaining included in 2 new STs that comprised 2 and 4 isolates each cluster (60.0%). These 6 new spoligotypes were already submitted for SITVIT database (www.pasteur-guadeloupe.fr:8081/SITVITDemo/).

Based on the spoligotypes, distinct families were identified: Haarlem (H), Latin American and Mediterranean (LAM), Undesignated (U), “T” family (modern TB strains), East-African Indian (EAI) and S lineage (Figure and Table 1).

Of the 83 *M. tuberculosis* isolates identified in the SITVIT database, 72 had classification into families and sublineages according to SpolDB4. The frequencies of these 72 isolates ranked in LAM, U, H and T families with 25 (34.7%), 17 (23.6%), 16 (22.2%) and 11 (15.3%) isolates respectively. The minor families observed in our study were EAI (n=1, 1.4%), S lineage (n=1, 1.4%) and H1-S (n=1, 1.4%).

Eleven *M. tuberculosis* isolates did not have classification into families according to SpolDB4 but they had an attributed ST number according to SITVIT database (ST2508, ST2512, ST2525, ST2563 and ST2654).

MIRU-VNTR typing

MIRU-VNTR typing was conducted in 84 clinical isolates and a total of 67 distinct MIRU patterns were obtained. Fifty-eight (69.0%) isolates were orphans and the remaining 26 (31.0%) were included in 9 clusters comprising 2 to 6 isolates each one (Figure).

Allele polymorphism analysis of twelve MIRU loci revealed that MIRU locus 40 was the most discriminatory locus with 8 alleles, followed by MIRU loci 23, 10 and 16. In the MIRU locus 40 the presence of 3 alleles was the most frequent followed by a single copy. MIRU 20, 26 and 31 loci were moderately discriminant. Other loci were less polymorphic with 2 alleles in MIRU loci 4, 20 and 39 and only 1 allele in MIRU locus 24, where a single copy was present in all 84 *M. tuberculosis* isolates analyzed (Table 2).

Combining spoligotyping and MIRU-VNTR typing

Within the 84 *M. tuberculosis* isolates analyzed by spoligotyping and MIRU-VNTR typing, 72 (85.7%) distinct genotypes were obtained, having 8 clusters with 100% similarity. Considering the similarity indices of at least 69%, we observed 3 distinct clonal groups, representing 92.9% of the isolates analyzed (Figure).

The data summarizing the discriminative power of each typing and combined methods by the Hunter-Gaston index are shown in Table 3.

Discussion

Molecular methods have been used for epidemiological studies of tuberculosis in some developed countries. However, this kind of study is scarce in developing countries. In Brazil, there are few studies of this nature.[2,24,25,26,27] The comprehension about TB pathogenesis, including differentiation between reactivation of latent infection and recent mycobacterial infection is important in developing prevention strategies.

To explore the TB molecular epidemiology in the northwest region of Paraná State, we chose to work with two effective and fast methodologies: spoligotyping and MIRU-VNTR typing. The clustering formation indicates exogenous infection and unique patterns of polymorphism are associated with reactivation of latent infection, indicating that primary infection occurred at different time and place, by different *M. tuberculosis* strains.[2]

A large diversity on circulation strains was observed in our study with *M. tuberculosis* isolates from low endemic TB setting in Brazil. The methodologies allowed 85.7% differentiation of the *M. tuberculosis* isolates analyzed, suggesting that TB in Maringá and other cities located in the northwest of Paraná State predominantly develops through reactivation of the latent infection. In agreement, Malaghini et al.[2] found a similar result in a recent study in Curitiba city, capital of Paraná State, with 93.5% differentiation of *M. tuberculosis* isolates using Mixed-linker PCR DNA fingerprinting. Cafrune et al.[25] analyzing *M. tuberculosis* isolates of three regions of Rio Grande do Sul State, south of Brazil, observed 66.0% clonal differentiation using IS*6110*-RFLP and spoligotyping. Borsuk et al. [24] analyzing isolates of two cities of Rio Grande do Sul State by IS*6110*-RFLP and spoligotyping observed high clonal diversity of *M. tuberculosis* isolates.

The distribution of 93 isolates analyzed by spoligotyping in our study showed 36 distinct patterns, which 6 (10.8% of isolates) have not been described in SITVIT yet. These new STs were submitted to this database by our TB work group. The LAM, H and T were the

largest families observed in our study and they are the three genotypic families most frequently found in Africa, Central America, Europe and South of America.[20]

Malaspina et al.[27] analyzing *M. tuberculosis* isolates from Araraquara, São Paulo State, southeast of Brazil, a state that borders Paraná, and Borsuk et al.,[24] from Rio Grande do Sul State, south of Brazil, reported the prevalence of ST53 (T1 sublineage). In our study, this ST was representative in only 5.4% (5/93) of the isolates and the ST46 (Undesignated – likely H lineage) was the prevalent, with approximately 14.0% (13/93) of the isolates.

One isolate belonged to EAI lineage (ST48, EAI1_SOM), which is more prevalent in South-East Asia, was detected in our study and in another study in the north of Brazil. The ST1892 (Undesignated lineage) and ST2512, characterized in our study, have exclusive geographic distribution in Brazil. ST61 (LAM10_CAM sublineage), ST563 (Undesignated lineage) and ST2654 identified in our study had not been described in our country yet. The other STs that have already been described in SITVIT database were identified in other studies in Brazil, but in our State it was the first time, probably because of the scarce study in our region (www.pasteur-guadeloupe.fr:8081/SITVITDemo/).

When MIRU-VNTR was applied to the study of *M. tuberculosis* isolates, the most allelic diversities were observed in MIRU loci 40, 23, 10 and 16 and moderate polymorphisms were found in MIRU loci 26, 20 and 31. Kovalev et al.[28] observed the most allelic diversities in MIRU loci 26, 31 and 10 in *M. tuberculosis* isolated in Ural region, Russian Federation. Sharma et al.[4] working with only six MIRU loci (MIRU 4, 10, 16, 26, 39 and 40) observed the most discriminatory loci were, in order of diversity, 26, 10, 16 and 40 in isolates from Kanpur, India.

The MIRU locus 24 was present as a single copy in all isolates analyzed in our study which came in agreement with Kovalev et al.[28] On the other hand, these authors refer to

MIRU locus 23 as having the second lowest discriminatory power which differs from our results that were the MIRU locus 39.

MIRU-VNTR really reduced the number of epidemiological links among isolates studied, that were overestimated by spoligotyping, thus demonstrating a better differentiation capacity of *M. tuberculosis* clinical isolates in our study. We believe that the use of newly 15 or 24-locus MIRU-VNTR[7,8,9] could increase the discriminatory power of this method.

Despite of the high discriminatory power of MIRU-VNTR typing observed in our study, there were isolates clustered by this method. In some cases, clustered isolates were discriminated by spoligotyping, demonstrating the need of the use of these two methodologies combined to provide a higher discriminatory power for epidemiological study. According to Cowan et al.[29] the combination of these two methodologies, the clustering rate is similar to that of IS*6110*-RFLP fingerprinting.

The results obtained with spoligotyping in our study suggest a possible epidemiological link between two patients, who were prisoners in the same prison during the same period of time, and presented the same molecular pattern (ST46). However, when these isolates were analyzed by MIRU-VNTR we observed that the *M. tuberculosis* isolates showed different patterns. In other *M. tuberculosis* isolates that were clustered (combining Spoligotyping and MIRU-VNTR typing), no correlation with retrospective epidemiological data was observed.

In summary, the present study offers the first insight about the genetic diversity of *M. tuberculosis* isolates from patients with TB in cities from northwest region in Paraná State. The data indicate that TB predominantly develops from reactivation of a latent infection. The combination of spoligotyping and MIRU-VNTR typing showed 85.7% clonal differentiation of the *M. tuberculosis* isolates analyzed in our study, showing to be highly discriminatory by Hunter Gaston index. Our results encourage additional studies in this setting in a well

designed prospective epidemiological study to be realized for evaluation of the National tuberculosis Control Plan.

Conflict of interest statement: No conflict of interest to declare.

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Table 1 Spoligotypes of the 9 *M. tuberculosis* isolates that MIRU-VNTR typing were not performed.

Identification n°	Octal Format															ST [a]	Lineage/ sublineage
85	7	7	7	7	7	7	7	7	7	7	6	0	7	7	1	53	T1
86	7	7	4	3	3	7	6	0	6	5	6	0	7	7	1	SND5	-
87	7	7	7	7	7	7	7	7	0	0	0	0	0	0	0	46	U (likely H)
88	7	7	7	7	7	7	7	7	7	7	2	0	7	7	1	50	H3
89	7	7	7	7	5	7	7	7	7	7	6	0	4	7	1	SND6	-
90	7	7	7	5	7	7	6	0	0	0	0	0	0	0	0	2508	
91	7	7	7	7	7	7	7	7	7	4	1	3	7	3	1	48	EAI1-SOM
92	5	7	7	7	7	7	6	0	7	5	6	0	7	7	1	SND4	-
93	7	7	7	7	7	7	6	0	7	5	6	0	7	7	1	64	LAM6

[a] ST: Shared International Type number according to SITVIT database.

SND: Spoligotype not described.

Table 2 Allelic polymorphism of 12 MIRU-VNTR loci from the 84 *M. tuberculosis* isolates from patients with TB in Maringá and region in the northwest of Paraná State, Brazil.

MIRU no.	Allele no.									HGI [a]	Conclusion
	0	1	2	3	4	5	6	7	8		
MIRU 2		1	10	72	1					0.254	Poorly discriminant
MIRU 4			82	2						0.047	Poorly discriminant
MIRU 10			6	14	40	23	1			0.673	Highly discriminant
MIRU 16	1	9	9	45	20					0.641	Highly discriminant
MIRU 20		21	63							0.380	Moderately discriminant
MIRU 23			1	16		29	35	3		0.678	Highly discriminant
MIRU 24		84								0.000	Poorly discriminant
MIRU 26				2	16	61	4	1		0.439	Moderately discriminant
MIRU 27		4	6	74						0.219	Poorly discriminant
MIRU 31		4	12	68						0.326	Moderately discriminant
MIRU 39		1	83							0.024	Poorly discriminant
MIRU 40		26	5	34	8	2	4	3	2	0.732	Highly discriminant

[a] HGI: Hunter-Gaston index.

The allelic diversity of the loci was classified as highly discriminant (HGI > 0.6), moderately discriminant ($0.3 \leq HGI \leq 0.6$) and poorly discriminant (HGI < 0.3), according to Sola et al. (2003).

Table 3 Discriminatory power of spoligotyping and MIRU-VNTR typing, alone and in association.

Methodologies	No. of distinct patterns	No. of clusters	No. of clustered isolates	HGI [a]
Spoligotyping	33	13	64	0.954
MIRU	67	9	26	0.991
Spoligotyping + MIRU	72	8	20	0.995

[a] HGI: Hunter-Gaston index.

CAPÍTULO III

CONCLUSÕES

A caracterização molecular de *Mycobacterium tuberculosis* isolados de pacientes com tuberculose pulmonar de nossa região

1. Foram detectados *spoligotypes* que ainda não foram descritos no Brasil e *spoligotypes* novos que ainda não foram descritos no banco de dados internacional SITVIT.

2. Dentre as duas técnicas de genotipagem de isolados de *M. tuberculosis* utilizadas, a técnica MIRU-VNTR foi a mais discriminatória.

3. Combinando as duas metodologias (*Spoligotyping* e MIRU-VNTR) obtêm-se uma melhor capacidade discriminatória dos isolados.

4. Dos isolados analisados pelas duas metodologias observamos 85,7% de diferenciação de *M. tuberculosis*, sugerindo que em Maringá e região a TB predominantemente desenvolve-se devido à reativação endógena de uma infecção latente.

PERSPECTIVAS FUTURAS

Esta informação inicial sobre a diversidade genética dos isolados de *M. tuberculosis* circulantes abre novos caminhos para o estudo da epidemiologia molecular da TB em nossa região. Posteriores estudos devem ser realizados com novos isolados de *M. tuberculosis* e analisando 24 *locus* na metodologia de MIRU-VNTR. A implantação de técnicas de biologia molecular permite a liberação de dados de diferenciação genética dos isolados e com isto conhecer melhor a dinâmica da transmissão dessa doença. O conhecimento dessa dinâmica de transmissão permite o desenvolvimento de estratégias de prevenção da TB.