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Genotipagem, perfil de resistência e análise de mutações dos isolados clínicos de *Mycobacterium tuberculosis* procedentes dos municípios brasileiros da tríplice fronteira Brasil/Paraguai/Argentina

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"Determinação coragem e autoconfiança são fatores decisivos para o sucesso. Se estamos possuídos por uma inabalável determinação conseguiremos superá-los. Independentemente das circunstâncias, devemos ser sempre humildes, recatados e despidos de orgulho."
*(***Dalai Lama)**

Genotipagem, perfil de resistência e análise de mutações dos isolados clínicos de *Mycobacterium tuberculosis* procedentes dos municípios brasileiros da tríplice fronteira Brasil/Paraguai/Argentina

RESUMO

A tuberculose (TB) é uma doença infecciosa cujo agente etiológico é principalmente *Mycobaterium tuberculosis*, acomete proporção significativa da população mundial e constitui um importante problema de saúde pública, principalmente em países em desenvolvimento. O estudo da epidemiologia molecular através de diferentes técnicas vigentes revolucionou a compreensão da epidemiologia da TB permitindo comparação entre linhagens e rastreamento de linhagens individuais. O presente estudo teve como objetivo analisar com o uso de técnicas moleculares (MIRU, MTBDR*plus* e RD^{Rio}), o fenômeno da transmissão da TB, perfil de resistência e análise de mutações e detecção da linhagem RD^{Rio} em pacientes com tuberculose pulmonar e extrapulmonar provenientes de municípios brasileiros e atendidos pela 9ª e 10ª Regionais de Saúde do Estado do Paraná, no período de julho de 2013 a junho de 2015. Foram identificados 97 isolados de *M. tuberculosis*, de pacientes de ambos os sexos e com idade variando de 15 a 77 anos. Os resultados estão apresentados no manuscrito: "Genotyping and resistance of *Mycobacterium tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina" em que a aplicação da técnica de 24 *loci* MIRU-VNTR diminuiu o número de isolados previamente agrupados por 12 *loci* MIRU-VNTR. A deleção RD^{Rio} foi encontrada em 16,49% dos isolados. Foram detectadas as mutações S531L e S315T nos genes *rpoB* e *katG*, que conferem resistência a rifampicina e isoniazida, respectivamente. Os resultados sugerem que a doença na região, na maioria dos casos, se deve a uma reativação de TB latente. No entanto, temos 4 *clusters* com 12 isolados que não foram possíveis de se diferenciar e uma possível relação epidemiológica é sugerida.

Palavras chave: Tuberculose. Epidemiologia molecular. *Mycobacterium tuberculosis.*

Genotyping and resistance of *Mycobacterium tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina

ABSTRACT

TB is an infectious disease whose etiologic agent is mainly *Mycobaterium tuberculosis* that affects a significant proportion of the world population and is an important public health problem, especially in developing countries. The study of molecular epidemiology through different existing techniques revolutionized the understanding of the epidemiology of tuberculosis allowing comparison between strains and tracking of individual strains. This study aimed to use molecular biology techniques such as MIRU, MTBDR*plus* and RD^{Rio} to try to understand more about the transmission of tuberculosis phenomenon, profile of resistance and analysis of mutations and detection of RD^{Rio} lineage in patients with tuberculosis pulmonary and extrapulmonary from Paraná State municipalities and attended by the 9^{th} and 10^{th} Health District, in the period July 2013 to June 2015. We identified 97 isolates of *M. tuberculosis* in patients of both sexes and aged 15-77 years. The results are presented in the manuscript: "Genotyping and resistance of *Mycobacterium tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina" in the application of 24 *loci* MIRU-VNTR technique, decreased number of isolates previously grouped by 12 *loci* MIRU-VNTR. The RD^{Rio} deletion was found in 16.49 % of the isolates. S315T and S531L mutations were detected in the *rpoB* and *katG* genes that confer resistance to rifampicin and isoniazid, respectively. The results suggest that TB in the region, in most cases, is due to a reactivation of latent TB. However, we have 4 clusters with 12 isolates that were not possible to differentiate and a possible epidemiological link is suggested.

Keywords: Tuberculosis. Molecular epidemiology. *Mycobacterium tuberculosis.*

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CAPITULO I

1.1 INTRODUÇÃO

1.1.1 A bactéria

O gênero *Mycobacterium,* único representante da família *Mycobacteriaceae* é constituído por bacilos curvos ou retos, com diâmetro de 0,2 a 0,7 µm e comprimento de 1 a 7 µm[1]. Devido à grande quantidade de lipídeos presentes em sua parede celular, especialmente os ácidos graxos de cadeia longa constituídos pelos ácidos micólicos, possuem propriedade de álcool-ácido resistência. São bactérias aeróbias, não esporuladas e classificadas de acordo com seu tempo de crescimento em, micobactérias de crescimento rápido quando requerem menos de sete dias, e de crescimento lento quando requerem mais de sete dias para detecção das colônias em meio sólido[2].

1.1.2 A doença

A tuberculose (TB) é uma doença infecciosa causada por bacilos do complexo *Mycobacterium tuberculosis (Mycobacterium tuberculosis*, *Mycobacterium bovis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium caprae e Mycobacterium canettii*) que continua sendo um dos maiores problemas de saúde pública do mundo[3]. *M. tuberculosis* foi descrito em 1882, por Robert Koch, e afeta predominantemente os pulmões, porém também pode acometer outros órgãos, sendo neste caso, classificada como TB extrapulmonar[3, 4].

A TB pulmonar é transmitida de pessoa a pessoa por via aérea, por gotículas de 1,0 a 5,0 µm de diâmetro produzidas pelo individuo portador da forma clínica pulmonar ou laríngea da doença, ao tossir, espirrar ou falar. Se o bacilo sobreviver às defesas primárias contra agentes infecciosos, se multiplica dentro do macrófago alveolar, onde iniciará o processo patológico[1,4]. Os bacilos podem, por mecanismos próprios, permanecerem viáveis, porém em um estado de latência no interior dos granulomas formados pela reação imunológica, durante toda a vida do indivíduo, reativam-se quando as condições são favoráveis ao seu desenvolvimento[1].

Essa doença crônica é caracterizada pela alta morbidade e mortalidade em países em desenvolvimento e áreas urbanas de países desenvolvidos. A doença acomete principalmente indivíduos na faixa etária correspondente a plena capacidade produtiva do indivíduo o que acarreta enorme prejuízo econômico ao país[2, 3, 5]. É estimado que 64 % dos casos de TB acometam homens e principalmente nos setores de mais baixa renda da população. Os fatores que intensificam o crescimento dessa doença são: os aglomerados humanos, a desnutrição, a deterioração das condições sócio-econômicas, medidas de controle ineficazes e também o aumento no número de casos de TB resistentes aos fármacos antituberculosos[6]. Algumas condições inerentes ao indivíduo contribuem para a redução da resposta à infecção e aumenta a probabilidade de TB ativa como: o uso de corticóides e outros fármacos imunossupressores, desenvolvimento de algumas doenças como diabetes mellitus e degenerativas e a infecção pelo vírus da imunodeficiência humana (HIV)[1].

A atual epidemia de TB está sendo sustentada principalmente por dois fatores: a infecção pelo (HIV) associada à doença ativa e o aumento da resistência dos isolados de *M. tuberculosis* frente aos fármacos antituberculosos mais efetivos[7].

1.1.3 Epidemiologia

Estima-se que em 2013, 9,0 milhões de pessoas desenvolveram TB, o que equivale a 126 a cada 100.000 habitantes e 1,5 milhões morreram da doença, dos quais 360.000 eram HIV-positivos. De acordo com a Organização Mundial da Saúde (OMS), um terço da população mundial deve estar infectada pelo bacilo, porém neste estágio não ocorre a transmissão da bactéria[1,3]. De 2000 a 2013 observou-se um declínio sutil dos índices da doença e a cada ano estima-se que 37 milhões de vidas são salvas por meio de diagnóstico e tratamento eficaz[3].

O Brasil está entre os 22 países que concentram o maior número de casos de TB mundialmente, com uma incidência populacional de 46/100.000 habitantes em 2013 e essa incidência pode variar significativamente de acordo com diferentes regiões geográficas[3,8]. O país, desde o início do século XX, vem adotando políticas públicas formuladas e implantadas pelos estados e organismos internacionais para o controle da TB. Em 1941 criou-se o Serviço Nacional de Tuberculose, marco efetivo do controle pelo Estado; fato relevante também foi a Campanha Nacional Contra a Tuberculose, em 1946. Porém, apenas no início década de 80 houve significativa redução da mortalidade e da incidência após introdução do esquema de

curta duração com rifampicina administrada ambulatoriamente. No entanto com o surgimento da AIDS o quadro da TB teve um retrocesso[9].

Em 1993, a OMS declarou que a TB se encontrava em estado de emergência mundial e foi recomendado o tratamento realizado pela estratégia DOTS (*Direct Observed Treatment Short-Course*). Este projeto da OMS preconiza a adesão política por parte das autoridades governamentais, estabelecimento de uma rede laboratorial de baciloscopia, garantia de medicação gratuita e de livre acesso, um sistema de informação adequado e a oferta de um tratamento supervisionado por profissional de saúde[10].

No estado do Paraná, a incidência da TB é de 18,7 casos/100.000 habitantes, porém em algumas regiões essa taxa é maior, como por exemplo, em Paranaguá (cidade portuária) e na tríplice fronteira entre Brasil/Paraguai/Argentina[11]. Os maiores municípios dessa região, Foz do Iguaçu no Brasil, Cidade do Leste no Paraguai e Porto Iguaçu na Argentina apresentam incidência de 56,6/100.000, 42,5/100.000 e 23,4/100.000, sendo uma região de alto risco de TB[12].

Foz do Iguaçu e Cascavel são os dois maiores municípios da região fronteiriça brasileira, apresentando índices de 56,6/100.000 e 25,5/100.000, respectivamente[8]. Essas altas taxas estão relacionadas às condições de vida da população, baixo acesso aos programas de saúde pública em algumas regiões, grande fluxo migratório temporal de pessoas nos três municípios próximos a divisa, a rápida travessia e o aumento das atividades econômicas e de turismo nos últimos anos. O grande números de turistas em visita às quedas d'água Cataratas do Iguaçu, uma das Sete Novas Maravilhas do Mundo, situada no Estado do Paraná-BR, fronteira com Missiones na Argentina, proporciona um aumento das atividades comerciais e como consequência um aumento populacional, o que favorece a transmissão de TB na região[8,12].

1.1.4 O tratamento da tuberculose

O tratamento quimioterápico padrão consite em associação dos fármacos, isoniazida (INH), rifampicina (RIF), pirazinamida (PZA) e etambutol (EMB), nos primeiros 2 meses e 4 meses de INH e RIF[13]. No Brasil, apesar da disponibilização gratuita dos medicamentos a taxa de cura ainda é baixa, 72 %, enquanto que a OMS preconiza, no mínimo, 85 %[3]. A disseminação dos isolados multidroga-resistentes (MDR) tornou-se uma grande ameaça aos programas de controle da TB. O projeto de abordagem para o gerenciamento dos isolados

MDR depende da correta identificação dos bacilos pertencentes a esse grupo e compreensão da dinâmica de transmissão[3].

Internacionalmente, são considerados MDR, os bacilos de *M. tuberculosis* que apresentam resistência simultânea a INH e a RIF. Mundialmente, a proporção de novos casos de TB multi-resistente (MDR-TB) foi de 3,5 % em 2013 em pacientes tratados pela primeira vez e de 20% nos casos com tratamento prévio[3]. A rápida detecção de resistência é essencial para o controle e tratamento da TB, reduzindo, assim, o custo do tratamento e a transmissão da doença. A resistência a estes fármacos se deve principalmente as mutações cromossomais nas regiões denominadas de *hot spot*. Até o momento, estudos tem mostrado que principalmente três genes estão relacionados a resistência a isoniazida (*katG, inhA e ahpC*) e um gene a resistência a rifampicina, o *rpoB*[14].

A identificação rápida destas alterações gênicas que podem estar relacionada com a resistência, são fundamentais no controle da disseminação da MDR-TB, sendo de interesse a aplicação de novas tecnologias como o teste desenvolvido pela Biomérieux, denominado GenoType MTBDR*plus*, que se baseia na tecnologia DNA-STRIP. Em 2009, Bwanga et al.[15] concluíram num estudo de meta-análise que a pesquisa direta de resistência à RIF e à INH em *M. tuberculosis* com a utilização do teste GenoType MTBDR*plus* permite a detecção imediata de MDR-TB com alta sensibilidade e especificidade. Arentz et al. (2013)[16] afirma, em estudo de revisão sistemática, que em áreas com alta prevalência de resistência à RIF e MDR-TB, os testes de rápida detecção podem ser um componente valioso de uma estratégia de controle da disseminação de MDR-TB.

1.1.5 Caracterização molecular do bacilo

A vigilância epidemiológica é um método de controle, eliminação ou erradicação das doenças e se realiza por meio de processos políticos, sociais e econômicos, com o uso de metodologia científica[1].

A epidemiologia molecular utiliza concomitantemente técnicas de biologia molecular, que caracterizam o conteúdo nucleotídico dos isolados de *M. tuberculosis* e a epidemiologia clássica, estuda a distribuição e os fatores determinantes da doença na população[1]. Em meados da década de 1980 foi relatada a primeira integração de métodos moleculares com epidemiologia clássica para discriminar isolados clínicos de *M. tuberculosis*[9].

O uso e aplicação de algumas técnicas laboratoriais de genotipagem têm contribuído como fonte de informação[10]. Estudos epidemiológicos moleculares demonstraram que

existem diferenças marcantes na apresentação da doença e demografia da população em países com baixa incidência e aqueles com alta de incidência de TB[17]. Em vários países africanos e asiáticos, as taxas de incidência são mais elevadas entre os jovens adultos, com a maioria dos casos resultantes de infecção recente ou reinfecção[18]. Por outro lado, em países de baixa incidência, Europa Ocidental e América do Norte, há uma maior proporção de casos de TB ativa em pacientes mais velhos ou entre os imigrantes de países de incidência elevada de TB[7].

Os objetivos práticos da epidemiologia molecular são identificar os micro-organismos responsáveis por doenças infecciosas, determinar sua rota de transmissão e relações filogenéticas e identificar os genes responsáveis por sua virulência, resistência a fármacos e produção de antígenos relacionados a vacinas[19]. Sendo assim, a tipificação molecular dos isolados de *M. tuberculosis* é importante para identificar isolados resistentes associados a surtos, resistência a fármacos e traçar a cadeia de transmissão. Com os resultados obtidos é possível também diferenciar os doentes com infecção remota reativada daqueles com infecção recente, contribuindo para os esforços de controle de TB regional[20, 21].

A genotipagem é usada primariamente para diferenciar entre transmissão recente e doença reativada, onde os isolados com o mesmo genótipo (mesmo *fingerprint*) são considerados "*clustered*", apresentam o mesmo padrão de ácido desoxirribonucleico (DNA) e são considerados epidemiologicamente ligados e resultantes provavelmente de uma infecção recente[8]. Porém, isolados com genótipos únicos podem ser considerados resultados de reativação de uma infecção latente presumidamente adquirida fora da população e do período de tempo de interesse[20-22].

Se um paciente tem TB recorrente (mais de um episódio de TB ativa) e os isolados iniciais e recorrentes estão disponíveis, é possível diferenciar entre recidiva (TB causada pela mesma cepa do episódio anterior) ou reinfecção (TB causada por uma cepa diferente)[20].

Metodologias previamente estabelecidas, como comparação de taxa de crescimento, morfologia da colônia, susceptibilidade a antibióticos selecionados e tipificação por fagos são úteis, porém a ineficiência discriminatória restringe sua aplicação na epidemiologia da TB[9]. O *fingerprinting* do DNA dos isolados de *M. tuberculosis* é uma ferramenta útil para estabelecer a extensão da transmissão recente numa população e os prováveis fatores de risco para transmissão recente; para identificar precocemente transmissões não suspeitas; para resguardar a transmissão de isolados resistentes a fármacos; e confirmar laboratorialmente transmissão cruzada[22, 23].

M. tuberculosis tem uma substancial variação genética que inclui uma ampla sequência de polimorfismos, em inglês *Large Sequence Polymorphisms* (LSPs) e *Single Nucleotide Polymorphisms* (SNPs), os quais são filogeneticamente informativos e úteis para análise genética[20]. A variação do número e localidade do elemento de inserção, (Insertion Segment - IS) 6110, as sequência repetitivas polimórficas ricas em guanosina-citosina (CRISPRs), e também o número variável de repetições aleatórias, *Variable Number Tandem Repeats* (VNTR), são variações frequentemente empregadas em epidemiologia molecular da TB[20].

A partir da descoberta de elementos altamente repetitivos no genoma de *M. tuberculosis* como marcadores para diferenciação de isolados, foram descritas técnicas que se utilizam de tais elementos, como por exemplo, polimorfismo de comprimento de fragmentos de restrição (RFLP-*Restriction Fragment Lenght Pylimorphism*) descrito por van Embden et al. em 1993[24], *Spacer Oligonucleotide Typing* (*Spoligotyping*) baseado na variação do *locus* DR descrito por Kamerbeek et al. em 1997[25] e MIRU-VNTRs (*mycobacterial interspersed repetitive units- variable number of tandem repeat)* descrita por Supply et al. em 2001[26].

A metodologia ideal para determinação do polimorfismo genético deve ser simples, acessível, ter um rápido tempo de resposta e os resultados devem ser num formato intercambiável entre diferentes laboratórios[20].

A tipificação usando RFLP-IS6110 tem sido considerada o padrão ouro para genotipagem de *M. tuberculosis* durante décadas. Porém, o método é trabalhoso, requer semanas de cultivo micobacteriano e purificação do DNA extraído, além de sofrer problemas de interpretação e portabilidade dos padrões complexos de bandas[22]. Além disso, quando os isolados possuem baixo (<6) número de cópias do IS6110, o poder discriminatório desta metodologia é muito baixo[22, 27].

A técnica *Spoligotyping* baseia-se no polimorfismo de DNA dentro do *locus* de repetição direta (*direct repeat* - DR *locus*) do complexo *M. tuberculosis*, determinando a presença ou ausência dos espaçadores específicos entre as sequências DR. A presença das sequências espaçadoras varia em diferentes isolados. A técnica é baseada na amplificação, *in vitro,* da região DR e segue-se subsequente hibridização diferencial dos produtos amplificados com oligonucleotídeos complementares às regiões espaçadoras variáveis localizadas entre as DRs (sondas) e imobilizados em uma membrana de Nylon (Biodyne C)[1,21]. Este *locus* apresenta alto polimorfismo, e a variação do número e identidade dos espaçadores dentro das regiões DRs é utilizada para diferenciar os isolados do complexo *M. tuberculosis* por reação em cadeia da polimerase (PCR), gerando diferentes spoligotipos. É uma técnica simples,

rápida, de baixo custo e os resultados gerados podem ser comparados com aqueles obtidos mundialmente[1, 21, 28].

Recentemente, em isolados pertencentes à família LAM do *Spoligotyping* foi identificado um genótipo, RD^{Rio}, no Rio de Janeiro, Brasil, que possui uma deleção de 10 genes (26.317 kb) ou aproximadamente 0,6% do genoma. Esta família tem sido responsável por aproximadamente 15% de todos os casos de TB mundialmente[29,30]. Esse genótipo pode estar associado a altas taxas de transmissibilidade e com casos mais severos de TB com alta carga bacilar[29,31]. A deleção RD^{Rio} foi detectada em estudos prévios por Lazzarini et al.[29] em 30 % das amostras no Rio de Janeiro, 37 % em Belo Horizonte[31] e 28 % em Rio Grande, sul do Brasil[32].

Atualmente, o método genotípico VNTR usando unidades repetitivas micobacterianas intercaladas, MIRU, parece ter o melhor potencial para substituição do RFLP. Essa técnica foi inicialmente baseada na variabilidade encontrada em 12 *loci* específicos intercalados em todo o genoma bacteriano[26,33]. Mais recentemente a genotipagem MIRU-VNTR empregando 15 ou 24 *loci* tem sido avaliada e aplicada para tipagem de *M. tuberculosis*[22]. Essa técnica é baseada na amplificação por PCR de múltiplos *loci* usando iniciadores específicos para as regiões de cada *locus* repetitivo e a determinação do tamanho dos produtos das amplificações em gel de agarose irá refletir o número de cópias do alvo MIRU-VNTR[22, 33]. Quando comparado os 24 *loci* pesquisados em relação aos 12 primeiramente propostos houve um aumento discriminatório de 40%, e de 23% quando combinado ao *Spoligotyping* entre isolados cosmopolitas, sob as mesmas condições[22]. Portanto, 24 *loci* MIRU-VNTR vêm sendo considerado uma ferramenta de alta resolução e de primeira linha para estudos filogenéticos[22].

Porém a combinação de MIRU-VNTR e *Spoligotyping* resultou no mais alto nível de discriminação para a maioria dos casos em larga escala, portanto essa combinação é atualmente a ferramenta mais útil a ser aplicada para genotipar *M. tuberculosis*[1,9,22]. Quando comparado MIRU-VNTR com RFLP-IS6110, os estudos mostram poder discriminatório semelhantes destas técnicas em isolados com alto número de cópias IS6110 e poder mais discriminatório do MIRU-VNTR em isolados com baixo número de cópias IS6110[27]. No entanto, em alguns casos nos quais os dados epidemiológicos, demográficos ou da investigação não estão disponíveis, é adequado o uso de RFLP-IS6110 para determinar a presença ou ausência de ligação entre os pacientes[22].

1.2 JUSTIFICATIVA

O estudo da variabilidade genética de linhagens bacterianas circulantes, bem como de resistência, possibilita o conhecimento da dinâmica da transmissão da TB na região de fronteira do Paraná com Paraguai e Argentina e tem apresentado considerados avanços pelo uso das técnicas de genotipagem. As técnicas baseadas na PCR como 24 *loci* MIRU-VNTR, são de fácil execução e de custo acessível e são ferramentas poderosas numa investigação de surtos de infecção hospitalar ou comunitária, bem como para diferenciar uma reinfecção exógena de uma reativação endógena e permitir a determinação do grau de relação entre os isolados obtidos de diferentes pacientes participantes do projeto. O monitoramento da linhagem RD^{Rio} em isolados clínicos por meio de técnicas baseadas na PCR poderá contribuir para elucidação da significância epidemiológica e a um melhor entendimento da dinâmica de transmissão. A rápida detecção de resistência é essencial para o controle e tratamento da TB, sendo de interesse a aplicação de novas tecnologias como teste GenoType MTBDR*plus*, reduzindo, portanto, o custo do tratamento e a transmissão da doença.

1.3 OBJETIVOS

1.3.1 Objetivo geral

Genotipar, avaliar o perfil de resistência e detectar mutações que geram resistência à isoniazida e rifampicina em *Mycobacterium tuberculosis* isolados de pacientes com tuberculose pulmonar e extrapulmonar provenientes de municípios brasileiros e atendidos na 9ª e 10ª Regionais de Saúde do Estado do Paraná, no período de julho de 2013 a junho de 2015.

1.3.2 Objetivos específicos

a) Caracterizar molecularmente os isolados de *M. tuberculosis* com a técnica 12 *loci* MIRU-VNTR;
b) Caracterizar molecularmente os isolados de *M. tuberculosis* com a técnica 24 *loci* MIRU-VNTR;

c) Detectar a frequência da linhagem RD^{Rio} entre os isolados;

d) Identificar a resistência e mutações gênicas relacionadas a resistência à rifampicina e/ou isoniazida por GenoType MTBDR*plus*;

e) Avaliar o grau de relação entre os isolados de *M. tuberculosis* analisados, obtidos de pacientes com TB, provenientes de municípios próximos à tríplice fronteira;

f) Determinar a diversidade alélica (HDGI) de cada *locus* do 24 *loci* MIRU-VNTR;

g) Ampliar o banco de dados de isolados de *M. tuberculosis* caracterizados molecularmente do setor de Bacteriologia Médica do Laboratório de Ensino e Pesquisa em Análises Clínicas (LEPAC/DAB/UEM).

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CAPITULO II

Manuscrito: "Genotyping and resistance of *Mycobacterium tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina"

Genotyping and resistance of *Mycobacterium tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina

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Abstract

Tuberculosis (TB) is an infectious disease whose etiologic agent is mainly *Mycobacterium tuberculosis,* is an important public health problem, especially in developing countries. This study aimed analyze with molecular biology techniques (24 *loci* MIRU, MTBDR*plus* and RD^{Rio} lineage) the genetic diversity of *M. tuberculosis* isolates from patients at Brazilian municipalities on the triple border Brazil, Paraguay and Argentina from July 2013 to June 2015. From the 97 *M. tuberculosis* identified, 24/95 (24.74 %) showed some resistance to the tested drugs, 17 were resistant to isoniazid (INH), five to streptomicym and two were multi-drug resistant (MDR). The resistant isolates showed a S531L mutation on *rpoB* gene and S315L mutation on the *katG* gene and 2 INH monoresistant showed no mutation to INH. The 24 *loci* MIRU-VNTR discriminated 89/97 (91.75 %) isolates. The RD^{Rio} deletion was detected in 16.49 % of the isolates. The results suggest that TB in the region, in most cases, is due to a reactivation of latent TB. However, we have 4 clusters included 12 isolates that were not possible to differentiate by 24 *loci* MIRU and a possible epidemiological link is suggested.

1. Introduction

Tuberculosis (TB) is a disease caused mainly by *Mycobacterium tuberculosis* and is one of the major global public health problems, especially in developing countries [1,2]. In 2013, the World Health Organization (WHO) estimated 9.0 million of TB cases globally, equivalent to 126 cases per 100,000 population. Brazil is among the 22 countries, which account for 80 % of all estimated incident cases worldwide [1]. Brazil had an incidence of 46/100,000 populations in 2013. However, this rate can vary significantly according to different geographical regions [1,3].

State of Parana, south of Brazil, had an incidence of 18.7/100,000 in 2013 and 0.9 mortality rate [4]. In some regions of the state, rates are higher, such as port area and on the triple border Brazil/Paraguay/Argentina. The municipalities in this region, Foz do Iguaçu in Brazil, Ciudad del Este in Paraguay and Puerto Iguazu in Argentina have a TB incidence of 56.6/100,000, 42.5/100,000 and 23.4/100,000, respectively [3,5]. TB control remains a priority in Brazil, thus a better understanding of TB transmission could help to identify risk settings as well as to improve contact tracing.

From the discovery of highly repetitive element in *M. tuberculosis* genome, techniques which use such elements as markers for strain differentiation were promptly described. The restriction fragment length polymorphism (RFLP) described by van Embden in 1993, Spoligotyping described by Kamerbeek in 1997 and MIRU-VNTR *(Mycobacterial Interspersed Repetitive Units-Vatiable Number Tandem Reapeats)* described by Supply in 2000 were largely used for this purpose [2,6,8]. Typing *M. tuberculosis* complex by IS*6110*-RFLP was considered the gold-standard during more than a decade. However, it is labor intensive and requires weeks for mycobacterial growth and purification of DNA extracted. Also, the IS*6110*-RFLP interpretation is sometimes difficult by the complex band patterns generated and has been a trend worldwide its replacement by other methods, such MIRU-VNTRs and Spoligotyping [7,8].

The 12 *loci* MIRU-VNTR method is fast, easy and is an alternative to IS6110-RFLP on epidemiological studies because its power discrimination is equivalent to IS6110 and permit easy and rapid comparison of results from independent laboratories [8,9]. However, 12 *loci* MIRU-VNTR has some limitations and it could be combined with an additional

genotyping method for greater accurancy [10]. It was recently defined a group of 24 *loci* MIRU-VNTR, including a subset of highly discriminatory 15 *loci* to be used as first-line tool in molecular typing of individual *M. tuberculosis* [7].

Recently was reported in Rio de Janeiro, Brazil, a predominant genotype, designed RD^{Rio}, that has a deletion of approximately 26,314 Kb, or 0.6 % of genome, resulting in loss of at least ten genes [11,12]. This genotype has been reported in 61 % of isolates in Rio de Janeiro, Brazil, which is a derivative of the Latin American Lineage (LAM) lineage [11,13]. This emerging genotype has been detected in other countries [11,14,15] and have shown to be a major cause of TB in the world and recently associated with more severe clinical forms of the disease with high bacterial load and high transmissibility [11]. Some research groups are relating the delation with isoniazid, pyrazinamide and streptomycin resistant [15,16]. To counteract several studies and expand these findings, detection of RD^{Rio} delation in the triple border aims to contribute to the recognition of the impact of this strain in the MDR transmission of the disease in the region.

The high migration on the border region (Brazil, Argentina and Paraguay), the strong regional trade, the closeness of the three cities and other little ones and the high TB incidence rates in this region justify an in-depth study through molecular techniques for differentiation and evaluates the evolution of TB in that setting [4]. The aim of our study was to characterize the genetic diversity of *M. tuberculosis* isolates from patients at Brazilian municipalities on the triple border Brazil, Paraguay and Argentina from July 2013 to June 2015, based on 24 *loci* MIRU-VNTR, detection of RD^{Rio} lineage and mutations associated with resistance to isoniazid and rifampicin.

2. Materials and Methods

2.1 Clinical isolates and drug susceptibility testing

The 97 *M. tuberculosis* clinical isolates included in this study were obtained from patients attended at the *Unidades Básicas de Saúde* (UBS) and public hospitals of the municipalities in the 9th and 10th Paraná Health Setting, south of Brazil, which also receive patients from the municipalities of Paraguay and Argentina borders. All isolates were sent to the *Laboratório Central do Estado do Paraná* (LACEN) for immunochromatography identification by TB Ag MPT64 Test BioeasyKit (Standard Diagnostic Inc 156-68, Hagal-

Dong, Giheung-Ku, Kyonggi-Do, Republic of Korea) and confirmation by MTB Q-PCR ALERT KIT (ELISABETH PHARMACON, SPOL.S R.O. BRNO, Czech Republic). The susceptibility testing to streptomycin (SM), isoniazid (INH), rifampicin (RIF) and ethambutol (EMB) was performed using the automated method BD BACTEC^{TM}MGIT^{TM}960 (BD, Franklin Lakes- NJ, USA) as recommended by the manufacturer's instructions.

The clinical isolates were sent to the *Laboratório de Ensino e Pesquisa em Análises Clínicas* (LEPAC/UEM) in Löwenstein-Jensen (BBL^{TM} - Becton Dickinson Microbiology Systems, Sparks, MD, USA) for performing molecular methodologies.

2.2 Genomic DNA extraction

The *M. tuberculosis* clinical isolates were cultured on Löwenstein-Jensen (BBL^{TM} - Becton Dickinson Microbiology Systems, Sparks, MD, USA) at 35 °C for 15 days. The DNA of the reference strain (*M. tuberculosis* H_{37}Rv) and clinical isolates were extracted using the QIAamp® DNA Mini Kit (QIAGEN Strasse 1, D-40724 Hilden, Germany) according to the manufacturer's instructions.

2.3 Mutation detection

The GenoType kit MTBDR*plus*/DNA STRIP (Hain GmbH Lifescience, Nehren, Germany) was used to detect the most common mutations in all INH and RIF resistant *M. tuberculosis* isolates previously detected by BACTEC^{TM}MGIT^{TM}960. DNA was extracted, amplified and detected via a hybridization and alkaline phosphatase reaction on a membrane strip, according to the manufacturer's instructions.

2.4 MIRU-VNTR genotyping

The 24 *loci* MIRU-VNTR was performed according to Supply et al. [7] and Santos et al. [17]. Firstly, the original 12 *loci* MIRU-VNTR (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40) were carried out as described by Supply et al. [7,8]. MIRU allele scoring was determined according to Mazars et al. [9] and Supply et al. [7]. After, 12 *loci* complementary (ETR-A, ETR-B, ETR-C, QUB-11b, QUB-26, QUB-4156, Mtub04, Mtub21, Mtub29, Mtub30, Mtub34 and Mtub39) were carried out for all isolates. Each *locus* was individually amplified using 1 μL of DNA (20 ng) in 24μL of a reaction mixture containing 1μL of the respective primers (10 μM) and PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA)

according to the manufacturer's instructions. PCR conditions for each primer pair are described by Santos et al. [17]. The amplified products were electrophoretically fractionated in 1.5 % (w/v) agarose gel using 1×TBE buffer at 100 Volts for 2 hours and visualized by transilumination and ethidium bromide staining. The 100-bp DNA Ladder (Invitrogen Life Technologies, São Paulo, Brazil) were used as molecular marker. The image was captured using a Vilber Quantum ST4 (M. H. Montreal Biotechnologies Inc., Dorval, Canada).

The results from each 24 *loci* MIRU PCR were combined to create 24-digit allelic profiles. The obtained data were inserted in the international database SITIVITWEB (http://goo.gl/aZaa36) for obtaining MIRU International Type (MIT) of each *M. tuberculosis* isolate. Dendrogram were constructed with the tools on the MIRU-VNTR plus website (http://goo.gl/Y0USKr) and clusters were obtained by Calculate Tree on the website. Clusters were defined as at least two *M. tuberculosis* isolates with identical patterns have been formed.

The discriminatory power of 24 *loci* MIRU-VNTR was calculated by the discriminatory Hunter-Gaston (HGDI) index [18].

2.5 RD^{Rio} analisys

Isolates belonging to RD^{Rio} lineage were identified by multiplex PCR according to Lazzarini et al. [11]. The PCR was performed in a final volume of 25 µL with 2 µL of DNA (20 ng), 0.4 µM of each primers (RDRioBrg and IS1561) and 19 µL of PCR Master Mix (Promega Corporation, Madison, Wisconsin, USA) according to the manufacturer's instructions. The amplification was performed in thermocycler (Applied Biosystems® Veriti® Thermal Cycler) programmed to: initial denaturation at 95 °C for 5 min; 45 cycles at 95 °C for 1 minute, 60 °C for 1 minute and 72 °C for 4 minutes and final extension at 72° C for 10 minutes. The amplified products were electrophoretically fractionated in 1.5 % (w/v) agarose using 1×TBE buffer at 100 Volts for 2 hours and visualized by transilumination and ethidium bromide staining. The 100-bp DNA Ladder (Invitrogen Life Technologies, São Paulo, Brazil) were used as molecular marker. The image was captured using a Vilber Quantum ST4 (M. H. Montreal Biotechnologies Inc., Dorval, Canada). Amplification of a unique 530 bp fragment was considered as a marker of Wild Type (WT) isolate. On the other side if the amplification rendered a unique 1175 bp fragment the isolate was classified as belonging to RD^{Rio} lineage. The appearance of two bands at 1175 bp and 530 bp could represent a mixed infection with a RD^{Rio} *M. tuberculosis* strain and a WT *M. tuberculosis* (RD^{Rio}/WT or mixed).

3. Results

Of 97 *M. tuberculosis* identified, 70 (72.2 %) were isolated from male patients which account for a 2.6 (M/F=70/27) male to female ratio. The age of patients ranged from 17 to 77 years old (mean ± SD: 36.23± 13.28), however 77/97 (79.4 %) of patients were between 20 to 60 years old.

Seventy-five percent (73/97) of the clinical isolates are susceptible to all the tested drugs (INH, RIF, EMB and SM) and 24/97 (24.74 %) clinical isolates showed resistance to one or more drugs; 17 were INH monoresistant, 5 SM monoresistant and 2 were multidrug-resistant (MDR) isolates (table 1 and 2).

A total of 19 resistant isolates were analyzed by using the genotype MTBDR*plus* assay. Both MDR isolates showed an amino acid change from serine to leucine in the codon 531 of *rpoB* gene and an amino acid change from serine to threonine in the *katG* gene. Of the 17 isoniazid monoresistant, 15 showed an amino acid change from serine to threonine at codon 315 of *katG* gene and two showed a wild type hybridization pattern in the *katG and inhA* genes by MTBDR*plus* assay.

The 12 *loci* MIRU-VNTR firstly performed on 97 *M. tuberculosis* isolates showed a total 70/97 (72.16 %) MIRU patterns and was discriminatory for 59/97 (60.82 %) isolates that showed unique MIRU pattern (Table 1). A total of 38/97 (39.17 %) isolates were in 11 clusters (I to XI), with two to nine isolates each (Table 2).

The largest cluster (IX) had 9 isolates and all of them were classified as orphans according to the international database SITVIT WEB (table 2). The second largest cluster was IV (MIT 519) have six isolates of patients from Foz do Iguaçu, Cascavel and Toledo, Parana, Brazil. Eleven of 59 isolates, with unique patterns, was classified as MIT 39, 45, 46, 117, 140, 180, 183, 184, 483, 565, 775 (table 1).

Table 1. Demographic, drug susceptibility testing, MIRU-VNTR typing, RD^{Rio} deletion and mutations related to isoniazid and rifampicin resistance in *M. tuberculosis* isolates not clustered from the triple border Brazil/Paraguay/Argentina.

Strain	City	Year	MIRU 12	MIT	MIRU 24	RD^{Rio}	Drug Susceptibility	Mutations MTBDR*plus*
BRF17	Foz do Iguaçu	2014	122225153324	Orphan	122225153424324354334234	WT	S	
BRF33	Foz do Iguaçu	2013	124221153223	Orphan	124221153323214444334211	WT	S	
BRF78	Foz do Iguaçu	2014	124226143424	Orphan	124226143424224235334132	WT	INH^R	S315T
BRF71	Foz do Iguaçu	2014	124326133225	Orphan	124326133525224264234232	WT	S	
BRF50	Cascavel	2013	124326133226	Orphan	124326133626224274414112	WT	S	
BRF70	Foz do Iguaçu	2014	124326133323	Orphan	124326133323323265422232	WT	INH^R	S315T
BRF85	Toledo	2014	124326143325	Orphan	124326143525224235444132	WT	INH^R	S315T
BRF18	Foz do Iguaçu	2012	124526153324	Orphan	124526153424224274224132	WT	S	
BRF23	Cascavel	2013	125326143324	Orphan	125326143424223235322132	WT	INH^R	S315T
BRF51	Foz do Iguaçu	2013	125326153224	Orphan	125326153424224264334132	RD	S	
BRF5	Cascavel	2012	125326153323	Orphan	125326153323224275234132	WT	S	
BRF22	Cascavel	2013	126313153223	Orphan	126313153323324474234333	WT	S	
BRF25	Foz do Iguaçu	2014	134325173334	Orphan	134325173434324264334223	WT	S	
BRF83	Foz do Iguaçu	2014	134326153222	Orphan	134326153222324274324132	WT	SM^R	
BRF78	Foz do Iguaçu	2014	223316163228	Orphan	223316163828323273422132	WT	S	
BRF87	Foz do Iguaçu	2014	224126153322	Orphan	224126153222224434434132	RD	SM^R	
BRF27	Foz do Iguaçu	2013	224136142321	Orphan	224136142121124364434132	RD	S	
BRF9	Foz do Iguaçu	2012	224223163121	Orphan	224223163121224484514132	WT	S	
BRF80	Foz do Iguaçu	2014	224225173321	Orphan	224225173121223253422133	RD	S	
BRF6	Cascavel	2013	224313153621	Orphan	224313153121323575332333	WT	S	
BRF79	Foz do Iguaçu	2014	224313163324	Orphan	224313163424224573334333	WT	S	
BRF8	Foz do Iguaçu	2013	224316143226	Orphan	224316143626224254434132	WT	S	
BRF29	Foz do Iguaçu	2014	224316153227	Orphan	224316153727123264534132	WT	S	
BRF56	Foz do Iguaçu	2014	224316153228	Orphan	224316153828214264434132	WT	SM^R	
BRF53	Foz do Iguaçu	2014	224316154228	Orphan	224316154828224254434122	WT	S	
BRF21	Foz do Iguaçu	2013	224316173228	Orphan	224316173828223234422132	WT	S	
BRF35	Foz do Iguaçu	2013	224326143226	Orphan	22432614362622228334132	WT	INH^R	no mutations
BRF57	Foz do Iguaçu	2014	224326173224	Orphan	124226143424224235334132	WT	INH^R	S315T
BRF47	Toledo	2013	225215163323	Orphan	225215163323324445324333	WT	INH^R	no mutations
BRF68	Foz do Iguaçu	2013	225225133323	Orphan	225225133323314645234333	WT	S	
BRF69	Foz do Iguaçu	2014	225313143332	Orphan	225313143232224565424333	WT	S	
BRF72	Foz do Iguaçu	2014	225313153222	Orphan	225313153222224454334333	WT	S	
BRF19	Foz do Iguaçu	2012	225326153325	Orphan	225326153525224255414132	WT	S	
BRF20	Foz do Iguaçu	2013	226313151323	Orphan	226313151323324665334333	WT	S	
BRF65	Foz do Iguaçu	2014	226314153323	Orphan	226314153323324564214333	WT	S	
BRF82	Foz do Iguaçu	2014	233325122325	Orphan	233325122525224554544233	WT	SM^R	
BRF74	Foz do Iguaçu	2014	233425153335	Orphan	233425153535313234322223	WT	S	
BRF66	Foz do Iguaçu	2014	324216143221	Orphan	324216143121313444422142	RD	S	
BRF58	Foz do Iguaçu	2014	324226183321	Orphan	324226183121224364434132	RD	S	
BRF91	Foz do Iguaçu	2014	124326143226	Orphan	124326143226224284334132	WT	S	
BRF95	Foz do Iguaçu	2014	233321153334	Orphan	233325153334324264314223	WT	S	
BRF97	Foz do Iguaçu	2014	225313152323	Orphan	225313152323323774222333	WT	S	
BRF98	Foz do Iguaçu	2014	233315153334	Orphan	233315153334324265314224	WT	S	
BRF99	Foz do Iguaçu	2014	225316143226	Orphan	225316143226224244714132	WT	S	
BRF104	Toledo	2015	223326153421	Orphan	223326153421324744224234	WT	S	
BRF105	Cascavel	2015	253131523233	Orphan	225313152323323264322333	WT	S	
BR0F6	Cascavel	2012	224313153621	Orphan	224313153121323574332333	WT	S	
BRF28	Foz do Iguaçu	2013	225225133323	Orphan	225225133323314644234333	WT	S	
BRF26	Toledo	2013	224326143324	39	224326143424224235314132	WT	INH^R	S315T
BRF44	Foz do Iguaçu	2013	225325153323	45	225325153323323765222333	WT	S	
BRF38	Foz do Iguaçu	2013	225325153324	46	225325153424324575234333	WT	S	
BRF15	Foz do Iguaçu	2012	224325143324	117	224325143424224254334234	WT	S	
BRF48	Foz do Iguaçu	2014	124326153224	140	124326153424223264334132	WT	S	
BRF54	Foz do Iguaçu	2014	124328153326	180	124328153626224365324132	RD	S	
BRF62	Foz do Iguaçu	2014	223326143323	183	223326143323324264214231	WT	S	
BRF73	Foz do Iguaçu	2014	225313153322	184	225313153222323564322333	WT	S	
BRF7	Cascavel	2012	125326153222	483	125326153222224274434132	WT	SM^R	
BRF89	Foz do Iguaçu	2014	124326163224	565	124326163424214224534132	WT	S	
BRF76	Foz do Iguaçu	2014	225325123323	775	225325123323224243334333	WT	S	

S: susceptible to streptomycin, isoniazid, rifampicin and ethambutol; SM^R: streptomycin resistant; INH^R: isoniazid resistant, RIF^R: rifampicin resistant; WT: Wild Type; RD: RD^{Rio} delation; S315T: amino acid change from serine to threonina at codon 315 of the *katG* gene; S531L: amino acid change from serine to leucine at codon 531 of the *rpoB* gene.

Table 2. Demographic, drug susceptibility testing, MIRU-VNTR typing, RD^{Rio} deletion and mutations related to isoniazid and rifampicin resistance in *M. tuberculosis* clustered isolates from the triple border Brazil/Paraguay/Argentina.

Strain	City	Year	MIRU 12	MIT	Cluster	MIRU 24		RD^{Rio}	Drug Susceptibility	Mutations MTBDR*plus*
BRF11	Foz do Iguaçu	2013	224226163321	26		224226163321224363514132	I a	RD	S	
BRF86	Foz do Iguaçu	2014	224226163321	26	I	224226163321224363334132	I b	RD	S	
BRF52	Foz do Iguaçu	2014	224226163321	26		224226163321222363434132	I c	RD	S	
BRF59	Foz do Iguaçu	2014	224226163321	26		224226163321224363434132	I d	RD	S	
BRF2	Foz do Iguaçu	2012	225313153323	42		225313153323313573322333	II a	WT	S	
BRF42	Foz do Iguaçu	2013	225313153323	42	II	225313153323323473324333	II b	WT	S	
BRF103	Foz do Iguaçu	2015	225313153323	42		225313153323313565222333	II c	WT	S	
BRF77	Cascavel	2014	225313153323	42		225313153323323563243413	II d	WT	S	
BRF12	Foz do Iguaçu	2012	224326133323	251	III	224326133323224463534211	III a	WT	INH^R+RMP^R	S531L and S315T
BRF45	Foz do Iguaçu	2013	224326133323	251		224326133323223464525211	III b	WT	INH^R+RMP^R	S531L and S315T
BRF4	Cascavel	2012	124326143324	519		124326143324224234334132	IV a	WT	INH^R	S315T
BRF75	Toledo	2014	124326143324	519	IV	124326143324224224214132	IV b	WT	INH^R	S315T
BRF100	Foz do Iguaçu	2014	124326143324	519		124326143324224234334132	IV c	WT	INH^R	S315T
BRF101	Toledo	2014	124326143324	519		124326143324224234334132		WT	INH^R	S315T
BRF36	Foz do Iguaçu	2013	124326143324	519		124326143324224234334132		WT	S	
BRF108	Toledo	2014	124326143324	519		124326143324224264434132	IV d	WT	INH^R	S315T
BRF32	Foz do Iguaçu	2013	224226143321	738		224226143321222244334142	V a	RD	S	
BRF55	Foz do Iguaçu	2014	224226143321	738	V	224226143321224243534142	V b	WT	S	
BRF14	Foz do Iguaçu	2013	123326143324	Orphan	VI	123326143324224233424132	VI a	WT	INH^R	S315T
BRF16	Foz do Iguaçu	2014	123326143324	Orphan		123326143324224234424132	VI b	WT	INH^R	S315T
BRF81	Foz do Iguaçu	2014	125326153122	Orphan	VII	125326153122224263334132		WT	INH^R	S315T
BRF84	Foz do Iguaçu	2014	125326153122	Orphan		125326153122224263334132		WT	INH^R	S315T
BRF1	Foz do Iguaçu	2013	224125152321	Orphan	VIII	224125152321114284534142	VIII a	RD	S	
BRF3	Foz do Iguaçu	2012	224125152321	Orphan		224125152321114284534142		RD	S	
BRF92	Foz do Iguaçu	2014	224125152321	Orphan		224125152321114284434142	VIII b	RD	S	
BRF49	Foz do Iguaçu	2014	233325153334	Orphan		233325153334322213434223	IX a	WT	S	
BRF61	Foz do Iguaçu	2014	233325153334	Orphan		233325153334324264314223	IX b	WT	S	
BRF94	Foz do Iguaçu	2014	233325153334	Orphan		233325153334324264314223		WT	S	
BRF96	Foz do Iguaçu	2014	233325153334	Orphan	IX	233325153334324264314223		WT	S	
BRF106	Foz do Iguaçu	2015	233325153334	Orphan		233325153334324264314223		WT	S	
BRF107	Foz do Iguaçu	2015	233325153334	Orphan		233325153334324264314223		WT	S	
BRF63	Foz do Iguaçu	2014	233325153334	Orphan		233325153334324264q314223	IX c	WT	S	
BRF64	Foz do Iguaçu	2014	233325153334	Orphan		233325153334324253312223	IX d	WT	S	
BRF102	Foz do Iguaçu	2015	233325153334	Orphan		233325153334324655214223	IX e	WT	S	
BRF13	Foz do Iguaçu	2013	333325173334	Orphan	X	333325173334324223424234	X a	WT	S	
BRF60	Foz do Iguaçu	2014	333325173334	Orphan		333325173334324264334223	X b	WT	S	
BRF10	Foz do Iguaçu	2012	234325153334	Orphan	XI	234325153434324234434223	XI a	WT	S	
BRF90	Foz do Iguaçu	2014	234325153334	Orphan		234325153334324264314223	XI b	WT	S	

S: susceptible to streptomycin, isoniazid, rifampicin and ethambutol; SM^R: streptomycin resistant; INH^R: isoniazid resistant, RIF^R: rifampicin resistant; WT: Wild Type; RD: RD^{Rio} delation; S315T: amino acid change from serine to threonina at codon 315 of the *katG* gene; S531L: amino acid change from serine to leucine at codon 531 of the *rpoB* gene.

The remaining 12 *loci* to complete 24 *loci* MIRU-VNTR typing was performed for all 97 *M. tuberculosis* isolates. This analysis was especially important for the clustered isolates by 12 *loci* MIRU-VNTR (Figure 1 and 2). The 24 *loci* MIRU-VNTR showed 89/97 (91.75 %) different patterns. The isolates in clusters I, II, III, V, VI, X, XI were completely differentiated. A total of 12/97 (12.37 %) isolates were maintained in four in clusters (IVc, VII, VIIIa and IXb) with two to five isolates (Table 2).

The analysis of allelic diversity for each of 24 *loci* MIRU (Table 3) revealed that MIRU *locus* 40 was the most discriminatory, followed by *locus* 31 with also eight alleles. The *loci* 10, 23, 26, 31, 40, QUB-26, Mtub 04, Mtub 21 and Mtub 30 were highly discriminatory

(HGDI ≥ 0.6), while *loci* 2, 4, 16, 20, 39, ETR-A, ETR-C, QUB-11b, QUB-4156, Mtub34 and Mtub 39 were moderately discriminatory (HGDI ≥ 0.3). *Loci* 24, 27, ETR-B and Mtub 29 had low discriminatory power (HGDI < 0.3). The *loci* 24 was the poorest power discrimination of all (HGDI=0.0).

The RD^{Rio} deletions were found in 16.49 % (16/97) isolates and the cluster I and VIII, classified according 12 *loci* MIRU-VNTR, were formed exclusively by isolates belonging to RD^{Rio} lineage. However, by 24 *loci* MIRU-VNTR, the cluster I was separated and the isolates were classified as 'orphans' and one isolate of the cluster VIII was excluded from the group. None of the isolates presented both WT and RD^{Rio} patterns.

4. Discussion

The present study is a molecular insight that was conducted with *M. tuberculosis* clinical isolates mainly from TB patients attended at the two largest cities in the Brazilian border, Foz do Iguaçu and Cascavel, with Argentina and Paraguay [3]. These high rates of TB in the border are related to people's living conditions, low access to public health programs in some regions, large temporal migration of people in the cities near the border, fast movement and the increase in economic activity and tourism in the last years among the three countries. The large numbers of tourists visiting the waterfalls of Foz do Iguaçu, one of the Seven New Wonders of the World, located in the State of Parana border with Misiones in Argentina provides an increase in commercial activity and result in a population increase, which favors transmission of TB in the region [3,5].

The age and gender of patients participating in our study (79.4 % were between 20 to 60 years old and male to female rate were 2.6) confirm previous studies carried out by Machado et al.[3], which found a predominance of TB among productive age and male people (72.9 % of all patients, and male to female ratio of 2.0) in this region. The disease distribution could be attributed to cultural barriers in small cities, where men are responsible for external activities, getting more exposed to TB [19]. The two (2.06 %) multidrug-resistant (MDR) isolates were from patients only from Foz do Iguaçu and this percentage is higher than those found in Parana (0.8 %) and Brazil (1.4 %) and lower than the world average (3.3 %) [1,4].

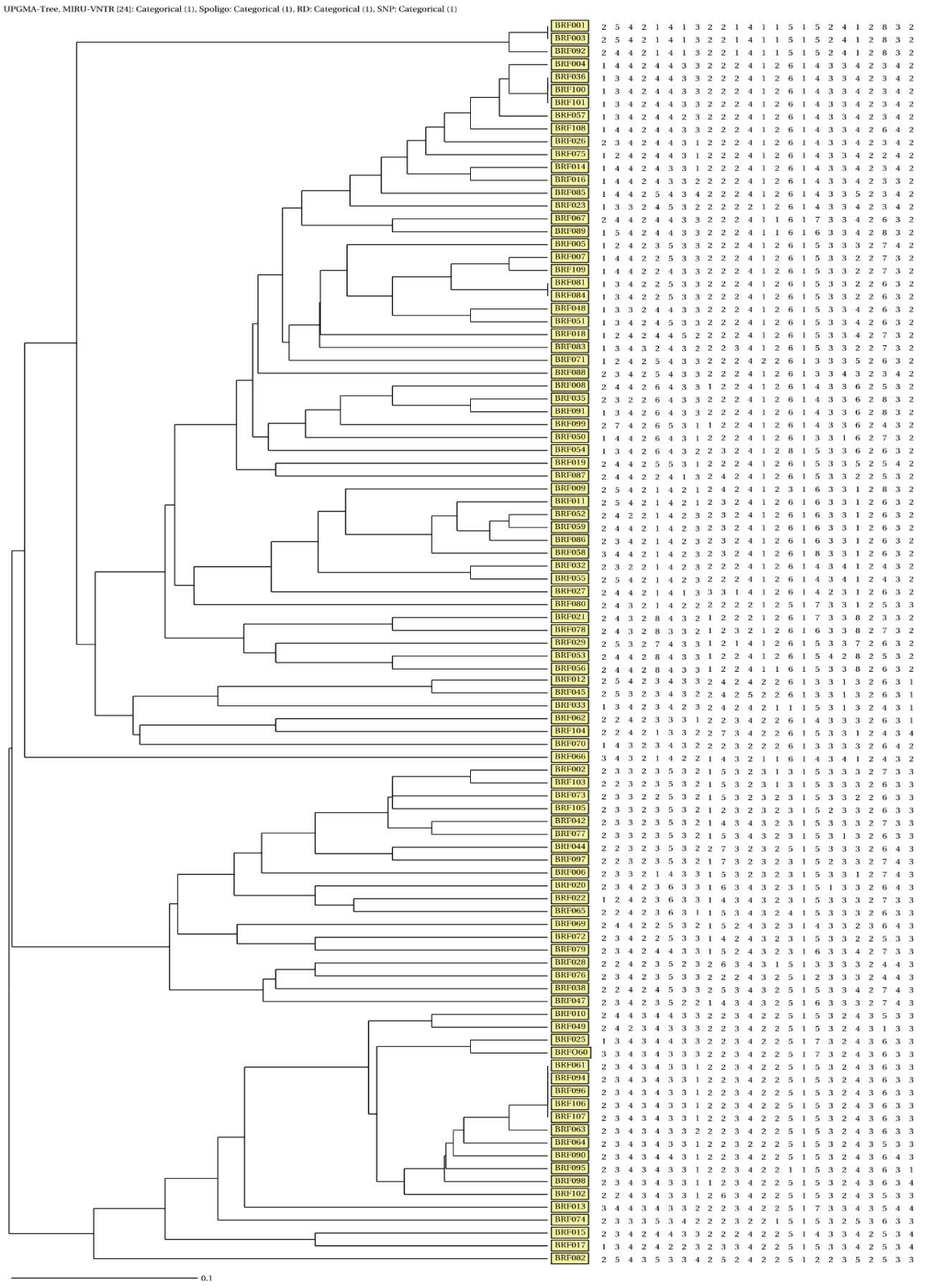

Figure 1: Combined numerical analysis of 97 *M. tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina using 24 *loci* MIRU-VNTR typing.

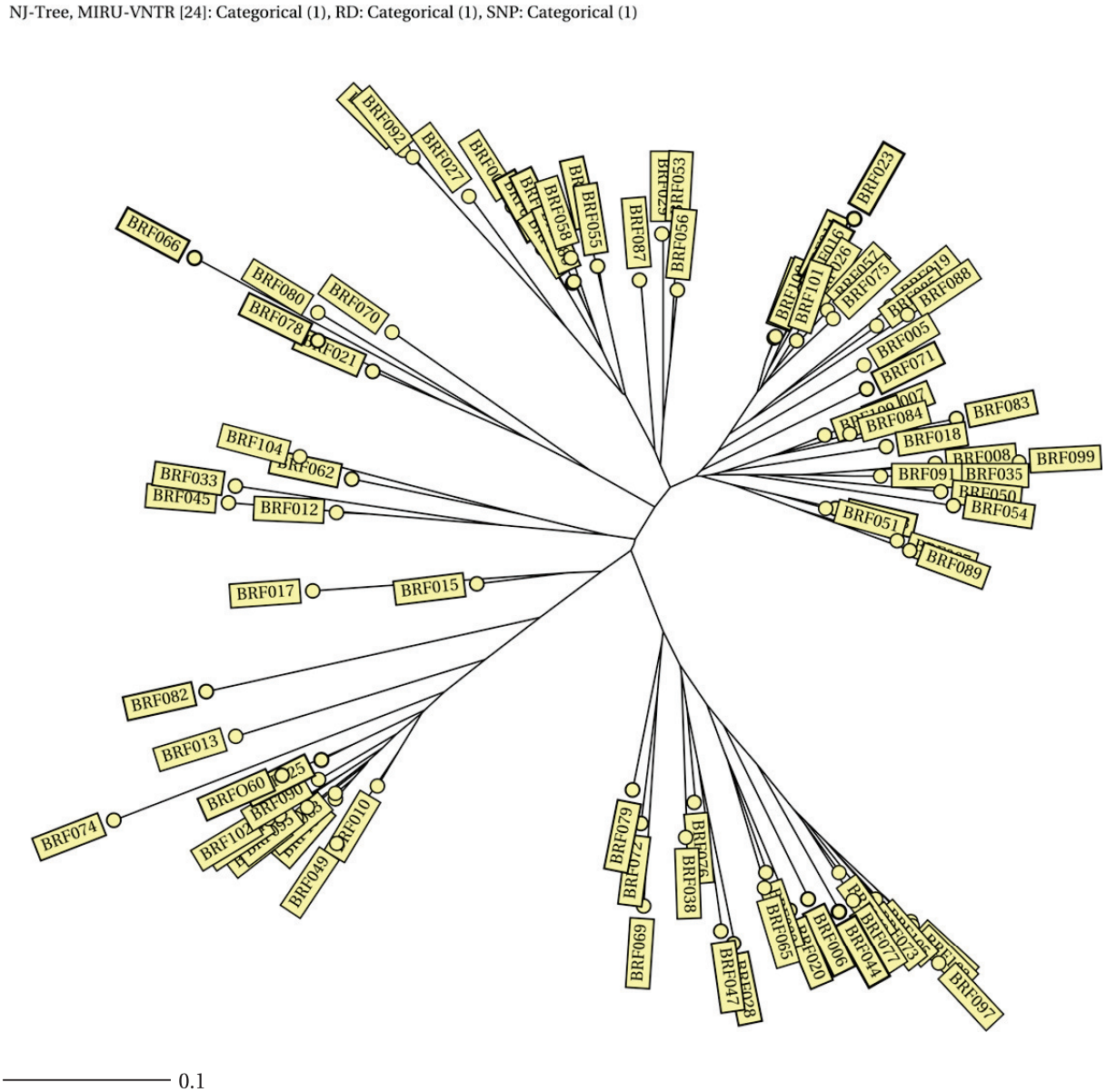

Figure 2. Radial tree combined numerical analysis of 97 *M. tuberculosis* clinical isolates from the municipalities of the triple border Brazil/Paraguay/Argentina using 24 *loci* MIRU-VNTR typing.

The rapid identification of genetic changes that can be related to the resistance is essential in controlling the spread of MDR-TB. Then, is of great interest to apply new technologies that are highly sensitive and specific to allow detect MDR-TB [20]. The MTBDR*plus* Kit identifies *M. tuberculosis* complex bacilli and detects mutations in three specifics genes: *rpoB,* which encode for the *β*-subunit of the RNA polymerase and confers resistance to RIF; *katG*, which encode for the catalase peroxidase and *inhA* regulatory region which encode for the NADH enoyl ACP reductase [22]. Mutation in specific region of *katG*

and *inhA* regulatory region can confers high and low-level resistance to INH, respectively [22,23].

Both MDR isolates showed no hybridization in the *rpoB* WT8 band and presence of *rpoB* MUT3 hybridization band on the membrane strip (MTBDR*plus* Kit), which represents a mutation and lead to an amino acid change from serine to leucine at codon 531 of the *rpoB* gene. According to Thakur et al. [23], Hillemann et al. [24] and Feliciano et al. [25] that studied isolates from north of India, Germany and southwestern Brazil, respectively, showed the most common mutation associated with resistance to RIF was S531L. However, ours MDR isolates showed S315T mutation at codon 315 of the *katG* gene.

In the 17 INH monoresistant isolates, 15 had S315T mutation. This is the most common mutation related to INH resistance. It was detected an absence of hybridization band at *katG* WT and presence at *katG* MUT1 in the membrane strip, which represents a mutation and amino acid change from serine to threonina at codon 315 of the *katG* gene. Thakur et al. [23] and Asante-Poku et al. [26] detected the same as the most common mutation associated with resistance to INH. On the other hand, Feliciano et al. [25] not detected this mutation as dominant. Two isolates, previously identified as INH monoresistant by the BACTEC^{TM}MGIT^{TM}960, showed a wild type hybridization pattern in the *katG* WT, *inhA*WT1, *inhA*WT2 and absence of hybridization with all mutation probes. Thus, the isoniazid resistance in these isolates is probably due to mutations on other genes, which are not included in the detection MTBDR*plus* Kit (mutation on *ahpC, kasA* or *ndh* gene) or other resistance mechanisms like an efflux mechanism [27].

The 12 *loci* MIRU-VNTR method is fast, easy to perform and is an alternative to IS6110-RFLP on epidemiological studies of TB and permit easy and rapid comparison of results from independent laboratories [8,9]. But, have some limitations, which can be overtake by combining with an additional genotyping method, as Spoligotyping or 24 *loci* MIRU-VNTR for greater accuracy [10].

It was recently defined a group of 24 *loci* MIRU-VNTR, including a subset of highly discriminatory of 15 *loci* to be used as first-line tool in molecular typing of individual *M. tuberculosis* [7]. The ideal method for determining the genetic polymorphism should be simple, affordable, have a quick response time and the results should be an interchange format to be analyzed between different laboratories [28].

Eighty-nine different MIRU patterns were detected in the 97 isolates by 24 *loci* MIRU-VNTR. Twelve isolates were into four clusters with two to five isolates each. It possibly may represent recently transmission between patients of each cluster, mainly in the eleven isolates that came from the same region (Foz do Iguaçu), while the 85 isolates with unique patterns represent reactivation of a latent infection presumably acquired outside the studied population [7,28]. Comparing the 12 *loci* MIRU-VNTR with 24 *loci* MIRU-VNTR, the discriminatory power varies significantly. While the first discrimination was 70/97 (72.16 %) isolates the second allowed 89/97 (91.75 %) isolates. The method showed a large number of isolates with unique patterns and we can have in mind a variety of strains circulating in the region.

The most allelic diversity observed in *M. tuberculosis* isolates studied was in the *loci* 40, which agree with Machado et al. [3] that conducted a study with patients from southwestern Parana, Noguti et al. [19] that conducted a study with patients from northwestern Parana, Sola et al. [29] that used a set of 116 *M. tuberculosis* clinical isolates from 11 different geographic origins and Soares et al. [30] that typed clinical isolates from Rio Grande, Rio Grande do Sul, Brazil. Others *loci* also consistent with the results of Sola et al.: *loci* 10, 23, 26, 31 and 40 have greater discriminatory power than the *loci* 4, 16 and 39, which showed moderate discriminatory power, while the *locus* 27 has low discriminatory power [29]. The *loci* 24 was the poorest power discrimination of all, same results are detected by Soares et al. [30] that observed too that *loci* 10, 16 and 40 were highly discriminatory, while *loci* 23, 26, 27 and 31 were moderately discriminatory and *loci* 2, 4, 20, 24 and 39 had low discriminatory power.

In a previous study carried out by Noguti et al. [19] with patients from northwestern Parana the most allelic diversity was observed in *loci* 40, 23 and 10 that matches with our results, and moderate polymorphism in *loci* 26, 20 and 31, which was different from observed in our findings in isolates in triple border.

Of the 97 isolates tested for the RD^{Rio} deletions only 16 (16.49 %) showed to have the deletion. In a study conducted by Soares et al. [30], in Southern Brazil with 45 clinical isolates, the percentage of this specific lineage was higher (n= 13, 28.9 %) than our and all of the isolates were classified as pertaining to the LAM family by Spoligotyping. Also, other authors have shown higher percentage than that found in our study, 30 % in Rio de Janeiro, RJ, 37 % in Belo Horizonte, MG, Brazil [11,31].

Table 3. Allele polymorphism of 24 *loci* MIRU-VNTR of 97 *M.tuberculosis* isolates triple border Brazil/Paraguay/Argentina.

	allele no.								HGDI	Conclusion
MIRU no.	0	1	2	3	4	5	6	7	8		
MIRU 2		30	63	4						0.4858	Moderate
MIRU 4			78	19						0.3183	Moderate
MIRU 10			1	19	52	22	3			0.6282	High
MIRU 16		5	15	75	1	1				0.3793	Moderate
MIRU 20		24	72	1						0.3918	Moderate
MIRU 23		2		14	1	28	51		1	0.6252	High
MIRU 24		97								0	Low
MIRU 26			2	7	23	49	9	6	1	0.6774	High
MIRU 27		1	7	88	1					0.1733	Low
MIRU 31		16	9	20	36	5	6	1	4	0.7837	High
MIRU 39			79	18						0.3054	Moderate
MIRU40		16	9	19	36	6	6	1	4	0.7867	High
ETR-A		5	53	39						0.5427	Moderate
ETR-B		12	85							0.2191	Low
ETR-C			4	18	75					0.3698	Moderate
QUB-11b			63	8	10	10	3	3		0.5539	Moderate
QUB-26		1	1	12	8	13	40	15	7	0.7685	High
QUB-4156				71	26					0.3965	Moderate
Mtub 04			14	44	28	10		1		0.6864	High
Mtub 21		20	25	50	2					0.6314	High
Mtub 29			15		81	1				0.2816	Low
Mtub 30		52	26	19						0.6087	High
Mtub 34			5	17	68	7				0.4749	Moderate
Mtub 39			5	52	35	5				0.5831	Moderate

The allelic diversity of the *loci* was classified as high discriminant (HGDI≥0.6), moderate discriminant (0.6>HGDI>0.3) and low discriminant (HGDI≤0.3), according to Sola et al. (2003).

In our study, two clusters (I and VIII) were composed only by RD^{Rio} isolates and all were from patients attended in Foz do Iguaçu, Brazil. However, by 24 *loci* MIRU-VNTR, the cluster I was separated and the isolates were classified as 'orphans' and one isolate of the cluster VIII was excluded from the group. This indicates that RD^{Rio} deletion could have erroneously been associated with recent transmission groups in studies that used only 12 *loci* MIRU-VNTR for genotyping.

The RD^{Rio} genotype is found in approximately 81.25 % of all LAM Family [30], which is responsible for 15 % of TB cases worldwide [13]. In a study conducted in the prison system of Rio de Janeiro in 2014 [32], the percentage of RD^{Rio} lineage *M. tuberculosis* was as high as 59.2 % and a significant clustering by Spoligotyping and IS6110-RFLP was observed. This high frequency has been associated with clinically severe form of disease, a more efficient transmission, virulence of the bacillus and with increased sputum bacillary load by the lineage [11,13,15,32]. This genotype has also been associated with isoniazid, streptomycin and pyrazinamide resistance in London, UK; New York, USA; southeastern Brazilian prison unit and Porto Alegre City, Brazil [15,16,32,33]. However, in our study it was observed that most of the isolates (15/16) were susceptible to all tested drugs. Only one was resistant to SM, which corroborate with other authors that not founded relation with drug resistance [34,35].

5. Conclusion

In conclusion, our study pointed out, despite the short study time (two years), the genetic variability and resistance profile of *M. tuberculosis* isolates circulating in the Brazilian cities bordering Paraguay and Argentina. These results integrate the start of a pioneer study on the dynamics knowledge of TB transmission in the triple border region. Our results showed a high clonal variability and we can have in mind a variety of circulating isolates in the region. It also suggests that TB, in most of cases, probably developed through reactivation of a latent infection. However, we have four clusters with 12 isolates that were not possible to differentiate and a possible epidemiological link is suggested mainly because they are living in the same region.

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Competing interests: None declared.

Ethical approval: The study was approved by the Ethics and Human Research Committee (COPEP) of the Universidade Estadual de Maringa, Parana (protocol No.409.166, CAAE No. 18314713.0.0000.0104) based on the requirements of Resolution Nº 466/12-CNS/MS. As no patients' information, which could to identify them were requested, the document participant consents achievement were exempted by the ethics committee.

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CAPITULO III

3.1 CONCLUSÕES

- O método 24 *loci* MIRU-VNTR revelou que, entre os 97 isolados de *Mycobacterium tuberculosis* obtidos de pacientes procedentes dos municípios brasileiros da tríplice fronteira Brasil/Paraguai/Argentina, 89 (91.75 %) diferentes padrões moleculares, sugerindo que a TB na região se deve possivelmente por reativação de infecção latente;
- A aplicação de 24 *loci* MIRU-VNTR reduziu o número de isolados previamente agrupados por 12 *loci* MIRU-VNTR. Doze pacientes permaneceram agrupados em 4 clusters, compreendendo de 2 a 5 isolados cada um;
- A análise do polimorfismo alélico quando aplicado a 24 *loci* MIRU-VNTR revelou que o *locus* 40 foi o mais discriminatório, com 8 alelos;
- A deleção RD^{Rio} foi encontrada em 16,49 % (16/97) das amostras entre os 97 isolados de *M. tuberculosis* obtidos de pacientes da região da tríplice fronteira Brasil/Paraguai/Argentina;
- Dois isolados multidroga-resistentes (MDR) apresentaram mutação no códon 531 do gene *rpoB* que leva a substituição de serina para leucina na subunidade *β* da enzima RNApolimerase. Apresentaram também mutações no códon 315 do gene *katG*, o que leva uma substituição de serina para uma treonina na catalase-peroxidase.
- Dos 17 isolados monoresistentes à isoniazida, 15 tinham mutações no códon 315 do gene *katG*, o que leva uma substituição de serina para uma treonina na catalase-peroxidase e 2 isolados não mostraram nenhuma mutação nos genes estudados.

3.2 PERSPECTIVAS FUTURAS

Este trabalho faz parte de uma pesquisa em andamento na região oeste do Paraná que é fronteiriça com o Paraguai e Argentina. O desenvolvimento de estudos posteriores envolvendo maior número de isolados de *M. tuberculosis*, com a inclusão de outras técnicas de biologia molecular, como o ERIC-PCR, *Spoligotyping* e aperfeiçoamento das técnicas de MIRU, utilizando cromatografia líquida ou detecção por fluorescência se faz necessário. Essas abordagens contribuirão para o conhecimento da dinâmica de transmissão da doença e permitirão o desenvolvimento de estratégias de prevenção da tuberculose nessa região.

TUBERCULOSIS

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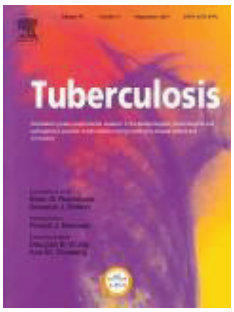

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