Original Article

Characterization of *Mycobacterium tuberculosis* Heteroresistance by Genotyping

Lida Jouca de Assis Figueredo[1], Isabela Neves de Almeida[1], Claudio José Augusto[2], Valéria Martins Soares[3], Philip Noel Suffys[4], Wânia da Silva Carvalho[5], Silvana Spíndola de Miranda[1]

[1]Mycobacteria Research Laboratory, Department of Internal Medicine, Faculty of Medicine, Federal University of Minas Gerais, [2]Mycobacteria Laboratory, Central Laboratory of Public Health of Minas Gerais, Ezequiel Dias Foundation, Minas Gerais, Brazil, [3]Júlia Kubitschek Hospital, Hospital Federation of the State of Minas Gerais, [5]Department of Social Pharmacy, Faculty of Pharmacy, Federal University of Minas Gerais, Minas Gerais, [4]Oswaldo Cruz Institute,Molecular Biology Laboratory Applied to Mycobacteria, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil

Abstract

Background: Heteroresistance is the coexistence of susceptible and resistant strains in the same individual, considered the preliminary step for total resistance, and can stem from mixed infection or clonal heterogeneity. The aim of this study was to evaluate the heteroresistance of *Mycobacterium tuberculosis* to rifampicin and isoniazid and its characterization. **Method:** GenoType MTBDRplus®; Sanger sequencing of the *rpoB*, *katG*, and *inhA* genes; and *Mycobacterial Interspersed Repetitive Unit – Variable Number Tandem Repeat* (MIRU-VNTR) were performed. **Results:** In a total of 654 isolates, 530 were resistant, 124 were susceptible, and 29 were heteroresistant to a first-line drug. GenoType MTBDR*plus*® detected heteroresistance in the *rpoB* gene in 26/29 (89.6%), as compared to 5/29 (17.2%) in the *katG* gene and 2/29 (6.8%) in the *inhA* gene. Four isolates showed heteroresistance in these genes. The Sanger sequencing detected heteroresistance in the *rpoB* gene in 7/29 (24.1%), as compared to 3/29 (10.3%) in the *katG* gene. In one isolate, heteroresistance was concomitant in both the *rpoB* and *katG* genes. MIRU-VNTR detected mixed infection in three heteroresistant isolates, while four isolates showed clonal heterogeneity. **Conclusions:** GenoType MTBDR*plus*® detected more cases of heteroresistance when compared to sequencing. It was also possible to characterize mixed infection and clonal heterogeneity by MIRU-VNTR.

Keywords: Heteroresistance, mixed infection, multidrug-resistant tuberculosis, *Mycobacterium tuberculosis*

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Introduction

In 2018, the incidence of tuberculosis (TB) in the world was 132/100,000 inhabitants, and the multidrug-resistant TB (MDR-TB)/resistant to rifampicin TB (RR-TB) incidence was 484,000/population. It was estimated that 3.4% of new TB cases and 18% of retreatment cases had MDR-TB/RR-TB.[1]

In Brazil, in the same year, it was estimated that there was an incidence of 1.2/100,000 inhabitants in reported cases of MDR-TB/RR-TB, with 1.5% among new cases and 8% among previously treated cases. Of this total, only 1119 MDR-TB/RR-TB cases and 26 extensively drug-resistant TB cases were confirmed in the laboratory.[1]

Rapid acquired resistance may be caused by late diagnosis, inadequate treatment regimen, or treatment abandonment, as well as by heteroresistance, which is the coexistence of susceptible and resistant bacteria in the same patient.[2,3]

Among the mechanisms that explain heteroresistance, a mixed infection is one of the most common, defined by the presence of strains with different patterns in two or more loci,[4] or clonal heterogeneity due to the division of a single lineage into susceptible and resistant clones due to biological evolution.[5]

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Address for correspondence: Prof. Silvana Spíndola de Miranda, Avenida Professor Alfredo Balena, 190 - Santa Efigênia, Belo Horizonte 30130-100, Minas Gerais, Brazil.
E-mail: silvanaspindola@gmail.com

ORCID:
Silvana Spíndola de Miranda: https://orcid.org/0000-0001-7245-4472

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The diagnosis of mixed infection is laborious and requires the strains to be individually identified from the original culture.[6]

The identification of *Mycobacterium tuberculosis* heteroresistance is underestimated, and GenoType MTBDR*plus*® (GenoType) and Sanger sequencing have been described as genotypic methods that can aid in this detection,[7-9] while the mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) is commonly used to detect mixed infection and clonal heterogeneity.[4]

The present study, therefore, sought to evaluate the heteroresistance of *M. tuberculosis* to rifampicin (RIF) and isoniazid (INH) using GenoType and sequencing, as well as the characterization of strains by MIRU-VNTR.

Method

Sample collection

The present study was performed in the Mycobacteria Research Laboratory of the School of Medicine of Federal University of Minas Gerais (FUMG) and included 654 clinical isolates of *M. tuberculosis* from the Júlia Kubitschek Hospital, a tertiary reference center; FUMG Clinical Hospital (FUMG), a high-complexity hospital; and the Mycobacterial Laboratory of the Ezequiel Dias Foundation/Central Laboratory of Public Health of Minas Gerais, from 2008 to 2017.

The treatment history and outcome of patients were obtained from the Notifiable Diseases Information System.

Drug susceptibility testing

Drug susceptibility testing was performed by the BACTEC™ MGIT™ 960 Systems by Becton Dickinson® (MGIT-960) at the Mycobacterial Laboratory of the Ezequiel Dias Foundation, as described by Siddiqi and Rüsch-Gerdes[10]

GenotypeMTBDRplus®

Molecular tests were performed in the Laboratory of Molecular Biology and Public Health, FUMG School of Pharmacy. The DNA was extracted from the colonies of clinical isolates in a solid Lowenstein–Jensen medium, using the GenoLyse® method, according to the manufacturer's instructions,[11] and a 5 µL of DNA was used for amplification by multiplex polymerase chain reaction (PCR) and reverse hybridization on the DNA strip.[11,12] The hybridized strip for all wild-type (WT) probes and nonhybridization for mutation probes proved to be susceptible. The absence of hybridization in WT probes or hybridization of any mutant probe indicates resistance, whereas the hybridization in WT and mutation probes indicates heteroresistance.[8,9]

Sanger sequencing

The sequencing of the *rpoB*, *katG*, and *inhA* genes was performed using the DNA extracted by GenoLyse,®[11] using primers as described by Dalla Costa *et al*.,[13] according to the following protocol: buffer 10x, $MgCl_2$ 50 µM, dNTP 2.5 µM, *primer forward* and *reverse* 20 pmol, Taq 5U, and 5 µL of DNA. PCR products were purified with polyethylene glycol 8000, and the Big Dye Terminator Kit v3.1, *forward* and *reverse primer* at 5 pmol, and 1 µL of the purified PCR product, was used.[13,14] The sequencing analysis was performed using the Applied Biosystems SeqScape® v2.5, Foster City, California, United States, and heteroresistance was determined when the sequencing simultaneously presented WT and mutation sequences.

Characterization of *Mycobacterium tuberculosis* heteroresistance by mycobacterial interspersed repetitive unit-variable number tandem repeat

The *M. tuberculosis* molecular characterization was based on 24 different *loci* containing MIRU-VNTR in fragments generated by manual monoplex PCR, as described by Supply *et al*.[15] Lineage identification of *M. tuberculosis* isolates was carried out using tree-based identification tools on the MIRUVNTR*plus* database (https://www.miru-vntrplus.org)[16] to analyze the presence of mixed infection or clonal heterogeneity in heteroresistant isolates. The mixed infection was defined by the presence of strains with different patterns in two or more loci, and clonal heterogeneity was determined when two distinct alleles in only one locus were identified.

Ethics

This study was approved by the FUMG Research Ethics Committee, logged under protocol number 122.941 (CAAE 06611912.8.0000.5149).

Results

Drug susceptibility testing

Of the 654 *M. tuberculosis* isolates, MGIT-960 identified 530/654 (81%) as susceptible and 124/654 (19%) as resistant, with 110/124 (88.7%) MDR-TB.

Genotype® MTBDRplus

Of the 654 isolates, 520/654 (79.5%) were susceptible and 134/654 (20.5%) were resistant when the GenoType was applied. A divergence was found in ten isolates, when compared to the MGIT; the results are shown in Table 1.

In this study, 84.3% (113/134) of the resistant's isolates were RIF resistant with mutations in the *rpoB* gene. By contrast, 90.6% (106/134) of the INH-resistant isolates presented mutations in the *katG* gene and 30.8% (36/134) in the *inhA* gene, while the concomitant mutations in the *katG* and *inhA* genes occurred in 21.3% (25/106) of the isolates.

Heteroresistance was observed in 4.4% (29/654) of the isolates. Heteroresistance to RIF was detected in codons S531 L and H526Y of the *rpoB* gene, while the INH heteroresistance was detected in codon C315T of the *katG* gene and in codon C15T of the *inhA* gene. In three isolates, heteroresistance occurred concomitantly in *rpoB* and *katG* genes and in one isolate in the *rpoB*, *katG*, and *inhA* genes [Table 2].

Sanger sequencing

The coexistence of WT sequences and mutations was detected by Sanger sequencing in 34.5% (10/29) of the isolates, which,

upon applying the GenoType, were detected as heteroresistant. The heteroresistance to RIF was detected in codons S531 L and S522A of the *rpoB* gene, while heteroresistance to INH was identified in codons C315T, G299C, and W293 L of the *katG* gene. In one isolate, the heteroresistance was simultaneous in the *rpoB* and *katG* genes [Table 3].

Characterization of *Mycobacterium tuberculosis* heteroresistance

MIRU-VNTR 24 loci was performed on the 29 isolates to distinguish whether heteroresistance originally occurred from mixed infection or clonal heterogeneity. Among the isolates, 3/29 (10.3%) presented mixed infection with two distinct alleles in two or more loci, confirming the presence of two different strains, and 4/29 (13.8%) isolates presented two distinct alleles in only one locus, confirming clonal heterogeneity [Table 4].

Another parameter analyzed in this study was the relationship of mixed infection and clonal heterogeneity with outcomes. Of the three patients with mixed infection, all were TB-MDR when analyzed by the MGIT-960, two with an outcome of abandonment and one with a cure.

Among the four isolates with clonal heterogeneity, two were TB-MDR and two were susceptible, when analyzed by the MGIT-960. Of these, three had a history of abandonment and one of cure [Table 5].

Patients that presented heteroresistant isolates changed the treatment in 25/29 (86.2%) cases, while 22/25 (88%) used several schemes. The outcome was a cure in 15/29 (51.7%) of the cases.

Discussion

Drug susceptibility testing

The conflicting results found in this study between resistant MGIT-960 and susceptible GenoType, may be due to mutations in regions that were not covered by the probes in GenoType.[17] The discordant results, in which susceptible MGIT-960 and resistant GenoType are explained by heteroresistance, reflect the slow evolution of the bacteria of a profile susceptible to resistance, which is not uncommon in *M. tuberculosis*.[18]

There is also the transmission of susceptible and resistant bacteria from patients who have not adhered to the treatment. These patients tend to harbor mycobacteria in genotypically detectable but phenotypically undetectable proportions.[2]

Heteroresistance, when associated with mixed infections of *M. tuberculosis*, causes an increase in the rate of false-negative drug resistance tests obtained by phenotypic susceptibility tests.[19-21] Therefore, heteroresistance can interfere in both the diagnosis and the treatment, which is more difficult to detect when using the phenotypic method from the preliminary stage to the total resistance.[2,3]

Molecular tests showed a low rate of heteroresistance, 29/654 (4.4%) when using GenoType and 10/654 (1.5%) when using Sanger sequencing. These results were similar to those reported in studies conducted in other regions, such as 3.7% (RIF) and 5.0% (INH) in Zimbabwe,[9] 1.4% in Italy,[22] and 1.9% in Finland and Russia.[23] However, there are studies that report higher rates, such as 14.3% in Uzbekistan[2] and 28.8% in India.[3] This discrepancy may be related to differences in detection methods or the prevalence of drug-resistant TB in each location.[2]

GenoType detected greater heteroresistance because the proportion of mycobacteria in the same isolate must be

Table 1: Phenotypic and genotypic results (*n*=134)

DST	MGIT-960 (%)	Genotype MTBDR*plus*® (%)
RIF_R, INH_R	110 (82.1)	96 (71.7)
RIF_S, INH_R	14 (10.4)	21 (15.6)
RIF_R, INH_S	0 (0.0)	17 (12.7)
RIF_S, INH_S	10 (7.5)	0 (0.0)

DST: Drug susceptibility test, RIF_R: Rifampicin resistance, INH_R: Isoniazid resistance, RIF_S: Rifampicin susceptible, INH_S: Isoniazid susceptible

Table 2: Heteroresistance using GenoTypeMTBDR*plus*®

Gene	WT probe	Mutant probe	Códon	MDR strain	Susceptible strain	*n*
rpoB	WT	MUT3	S531L	21	2	23
	WT	MUT2A	H526Y	2	0	2
	WT	MUT2B	H526D	0	1	1
katG	WT	MUT1	S315T	3	3	6
inhA	WT	MUT1	C15T	1	1	2

MDR: Multidrug resistance

Table 3: Heteroresistance using Sanger sequencing

Gene	Codon	MDR strain	Susceptible strain	*n*
rpoB	S531L	5	1	6
	S522A	0	1	1
	C315T	1	1	2
katG	G299C	1	0	1
	W293L	1	0	1
inhA	C15T	-	-	-

MDR: Multidrug resistance

Table 4: Characterization of heteroresistance using mycobacterial interspersed repetitive unit-variable number tandem repeat (*n*=7)

Characterization	MIRU 16	ETR A	Mtub 21	Mtub 29	Mtub 30	Mtub 39
Mixed infection	3	3	3+4	4+5	1+5	2+3
	1	2	2+3	4	2+1	3+7
	0	2+1	3	4	1	2+3
	3	2	3+2	4	1	2
Clonal heterogeneity	2+0	2	2	3	2	2
	2+0	2	3	4	1	3
	2+3	1	3	4	1	3

MIRU: Mycobacterial interspersed repetitive unit, ETR: Exact tandem repeat, MTUB: *Mycobacterium tuberculosis* loci

Table 5: Phenotypic test, outcome, and characterization of mixed infection and clonal heterogeneity using mycobacterial interspersed repetitive unit-variable number tandem repeat (*n*=7)

Characterization	Locus (*n*) variation	MIRU-VNTR	Outcome	MGIT-960
Mixed infection	4	Ugandal/Haarlem	Abandonment	MDR
	3	Ugandal/Haarlem	Abandonment	MDR
	2	LAM/Ugandal	Cure	MDR
Clonal heterogeneity	1	LAM	Abandonment	MDR
	1	LAM	Abandonment	MDR
	1	LAM	Abandonment	Susceptible
	1	LAM	Cure	Susceptible

LAM: Latin-American-Mediterranean, MDR-TB: Multidrug-resistant tuberculosis

95% susceptible and 5% resistant, as compared to Sanger sequencing, in which 50% is susceptible and 50% is resistant, as reported by Folkvardsen *et al*.[7]

In detecting heteroresistance, GenoType showed the absence of certain probes that were detected by Sanger sequencing, requiring the incorporation of additional probes.[8,12,24]

When heteroresistance is detected in GenoType or Sanger sequencing, the choice of appropriate treatment is complex. Therefore, it is necessary to interpret the genotype data together with the clinical data of the patients,[25] taking into account the locations of the mutations and determining whether or not they are related to low or moderate level of resistance through quantitative tests, such as the minimum inhibitory concentration.[26]

Characterization of *Mycobacterium tuberculosis* heteroresistance

Mixed infections and heteroresistance may be particularly common in regions with a high TB rate, especially MDR-TB,[3,6,27] and they can aid *M. tuberculosis* strains in the acquisition of mutations, facilitate the spread of drug-resistant strains, and increase the rate of treatment failure.[17,24] MIRU-VNTR is an important tool and has been used to assess whether mixed infections contribute to heteroresistance.[27]

Heteroresistance caused by clonal heterogeneity infection occurs due to the segregation of susceptible and resistant organisms.[5] Numerous reports on selective pressure in inadequate treatments have been described in prior literature,[28] as well as in the present study, in which these patients showed a history of abandonment and several previous treatments.

Conclusions

GenoType MTBDR*plus*® detected more cases of heteroresistance when compared to sequencing, and it was possible to characterize mixed infection and clonal heterogeneity by MIRU-VNTR.

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Conflicts of interest

There are no conflicts of interest.

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