Article/Artigo

Drug resistance of *Mycobacterium tuberculosis* strains in Southern Brazil

Estudo da resistência de cepas de *Mycobacterium tuberculosis* aos antimicrobianos no Sul do Brasil

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ABSTRACT

Introduction: The aim of this work was to evaluate the prevalence of *Mycobacterium tuberculosis* (MT) strains with mutations that could result in resistance to the main drugs used in treatment in a region with one of the highest numbers of tuberculosis (TB) cases in southern Brazil. **Methods**: Deoxyribonucleic acid (DNA) from 120 sputum samples from different patients suspicious of pulmonary tuberculosis who attended the Municipal Public Laboratory for *Mycobacterium sp*. diagnosis was directly amplified and analyzed by PCR-SSCP. The DNA was amplified in known hotspot mutation regions of the genes *rpoB, ahpC, embB, katG, inhA*, and *pncA*. **Results**: The percentage of samples positive by culture was 9.2% (11/120); 5% (6/120) were positive by bacilloscopy and MT-PCR, and DNA fragments of the aforementioned resistance genes could be amplified from seven (7) of the eleven (11) samples with positive results, either by culture or PCR/bacilloscopy. All presented a SSCP pattern similar to a native, nonresistant genotype, with the ATCC strain 25177 as control, except for one sample (0.01%), which presented a SSCP profile demonstrating mutation at the *embB* gene. **Conclusions**: These results are consistent with the empirical observations by physicians treating TB patients in our region of a low occurrence of cases that are refractory to conventional treatment schemes, in contrast to other parts of the country. Continued surveillance, especially molecular, is essential to detect and monitor the outbreak of MT-resistant strains.

Keywords: Antibiotic. *Mycobacterium*. PCR-SSCP. Resistance. Tuberculosis.

RESUMO

Introdução: O objetivo deste trabalho foi avaliar a prevalência de cepas de *Mycobacterium tuberculosis* (MT) com mutações que podem resultar em resistência às principais drogas utilizadas no tratamento em uma das regiões com o maior número de casos de tuberculose (TB) no Sul do Brasil. **Métodos:** O ácido desoxiribonucleico (DNA) de 120 amostras de escarro de diferentes pacientes com suspeita de TB pulmonar que procuraram o serviço público de saúde do município sede da região para o diagnóstico de MT foi diretamente amplificado e analisado por PCR-SSCP. Foram amplificadas regiões conhecidas onde ocorrem a maioria das mutações nos genes *rpoB, ahpC, embB, katG, inhA*, and *pncA*. **Resultados:** Nove vírgula dois por cento (11/120) das amostras apresentaram resultado positivo por cultura, 5% (6/120) foi positiva por bacilscopia e PCR para MT, e os fragmentos dos genes mencionados puderam ser amplificados em sete (7) dos onze (11) casos com resultado positivo, seja por cultura ou PCR/baciloscopia. Todos estes casos apresentaram um padrão de SSCP similar ao genótipo nativo, não resistente, por comparação com a cepa controle ATCC 25177, com exceção de uma amostra (0,01%), que apresentou um padrão de SSCP mutante no gene *embB*. **Conclusões:** Estes resultados são consistentes com as observações empíricas por parte dos clínicos que tratam os pacientes com TB na região, de uma baixa ocorrência de casos refratários ao tratamento convencional, em contraste com outras partes do país. Porém, a vigilância contínua, especialmente molecular, é essencial para identificar e monitorar o aparecimento de cepas de MT resistentes.

Palavras-chaves: Antibióticos. *Mycobacterium*. PCR-SSCP. Resistência. Tuberculose.

INTRODUCTION

Because tuberculosis (TB) has deep social roots intimately linked to unequal income distribution, the problem with the disease reflects the level of development of a country. Causes related to poverty, the weak organization of health systems, and administration deficiencies limit technology implementation and, as a consequence, contribute to the dissemination of the etiological agent and the increase in population susceptibility. Since 1993, the World Health Organization (WHO) has considered TB a global emergency, thus supporting measures for its control[1,2]. However, the battle against the disease is far from over.

The region of the Itajaí Valley in the State of Santa Catarina, which has about 500,000 inhabitants, is among the regions with the highest number of TB cases in southern Brazil (source: http://portalses. saude.sc.gov.br).

Reports of *Mycobacterium tuberculosis* (MT) strains resistant to the antibiotics used in treatment have been noted everywhere in the world[3]. Besides empirical observations of the absence of resistant *Mycobacterium* strains in our region, reported by the physicians who treat the TB patients, it is obviously important to offer and perform active research to monitor an eventual rise in resistance using fast and efficient tools, such as molecular methods. As is well known, MT cultures may take up to 8 weeks to yield results, and in the case of positivity, antibiotic sensitivity testing by traditional microbiologic techniques takes about 4 weeks more. During this time, the patient may already be spreading MT-resistant strains to close contacts. Using methods capable of offering results in a couple of days allows physicians to be readily informed and to take the appropriate therapeutic measures on time. Based on empirical information about the observation, obtained from the Municipality Laboratory staff, the number of patients with more than one year of TB treatment still presenting positive bacilloscopy results may be significant.

In the State of Santa Catarina, as well as in most of the country, *M. tuberculosis* susceptibility testing is currently performed only at the Central Public

Laboratory in the capital of each state by microbiologic methods. It is interesting to note that before 2009, the guidelines accepted by the public health system allowed requests for these tests only by patients with certain conditions, including HIV patients, symptomatic patients with negative bacilloscopy, and any patient who is not responsive to current therapy, among other special circumstances[4]. An immune competent patient eventually infected by a resistant MT strain had to wait for treatment failure and 8 more weeks on average for laboratorial evidence that could improve his outcome. Only after the implementation of new national guidelines, published in August 2009, has culture with antibiotic sensitivity testing for any respiratory symptomatic patient with suggestive radiographic findings been advised[5]. However, this is not the case observed in clinical practice, which continues to be based on bacilloscopy findings for primary diagnosis.

In this context, through the Clinical Laboratory of the University Ambulatory Center of our institution, which assists patients in the whole region as a reference service, we aimed to offer the possibility of detection of mutations that may lead to MT resistance to first-line oral antibiotics used for TB treatment in Brazil, through a molecular method such as polymerase chain reaction followed by single-strand conformational polymorphism (PCR-SSCP). This methodology is a robust, fast, and reliable technique that may allow a medical decision in up to a couple of days. Furthermore, to date, no updated information about the prevalence of *Mycobacterium*-resistant strains is available in our region. As a starting point, in this study we have evaluated the eventual presence and prevalence of resistant TB strains in our public health system (SUS) with the use of PCR-SSCP. These results may contribute to the evaluation of the current TB treatment scheme used in the system, as well as provide new information about the prevalence of resistant strains in our population. Such information is crucial for evaluating the management and surveillance policies on the tuberculosis control effort.

METHODS

Patients and samples

We analyzed 120 sputum samples from different patients suspicious of pulmonary tuberculosis who have attended the Municipal Public Laboratory for *Mycobacterium sp.* diagnosis. After processing for bacilloscopy at the site, the samples were sent to the Clinical Laboratory of the University Ambulatory of our institution for culture and PCR.

Sample treatment

The samples were treated by a conventional modified method using 1% NaOH, SDS, and phosphoric acid[6]. The treated samples were then used for culture and PCR. DNA was extracted and purified as described elsewhere[7].

Mycobacterium tuberculosis diagnosis

Bacilloscopy was performed directly on sputum sample slides stained by the Ziehl-Neelsen method. The culture was prepared by inoculation of samples in Löwenstein-Jensen medium and incubation at 37ºC for up to 8 weeks. For PCR, DNA was amplified with a primer pair specific to *M. tuberculosis* (MT1: 5'-CCT.GCG.AGC.GTA.GGC.GTC.GG-3' and MT2: 5'-CTC.GTC.CAG.CGC.CGC.TTC.GG -3'), resulting in a 123bp fragment of the insertion element *IS*6110[8]. Positive samples obtained by any of the methodologies used for MT diagnosis were used in PCR-SSCP tests.

Polymerase chain reaction

For detection of MT strains harboring mutations that may lead to resistance to rifampicin, isoniazid, pyrazinamide, and ethambutol, we amplified hotspot sites at the genes *rpoB*, *katG*, *inhA*, *ahpC*, *pncA*, and *embB*. The amplicons were later submitted to SSCP-PCR (single-strand conformation polymorphism-polymerase chain reaction)[9]. The primers were synthesized according to the sequences described by others[10]. Amplification reactions were performed in a volume of 25uL, consisting of 0.5uL of each 10uM primer (Invitrogen), 0.2uL of 100uM dNTPs (Invitrogen), 1.0uL of 50mM $MgCl_2$ (Invitrogen), and 1.0U of Taq DNA polymerase in the provided reaction buffer (Invitrogen). The annealing temperatures were 64ºC for the *rpoB* and *embB*, 57ºC for the *pncA*, and 55ºC for the *ahpC*, *katG*, and *inhA* genes. As naïve control, purified DNA from the ATCC 25177 *M. tuberculosis* strain was amplified.

Single-strand conformation polymorphism

The PCR amplicons of the aforementioned gene hotspots were resolved by 1% agarose gel electrophoresis with ethidium bromide staining under UV light. Those samples with positive amplification were submitted to native 5.5% polyacrylamide gel electrophoresis after denaturation of the double-strand DNA. Briefly, 18µL of formamide dye mix was added to 2µL of each PCR product, followed by boiling for 5min and immediate cooling on ice; 2µl of 10x sucrose gel loading buffer was added, after which the mixture was applied to the gel and submitted to 300V (45mA) for 5h. The gel was stained with $AgNO_3$ and photographed[11].

Ethical considerations

This project was approved by the Committee on Ethics in Research with Human Beings of our institution (Protocol no. 032/03) under the guidelines of the Helsinki Declaration of 1964 and further revisions.

RESULTS

Among the 120 patient samples tested for MT diagnosis, six (5%) were positive either by bacilloscopy or PCR with primers specific to the *M. tuberculosis IS*6110 element. On the other hand, eleven (9.2%) were positive by culture.

The samples that were positive by either method were tested by PCR-SSCP for detection of mutations in the hotspot regions of the genes responsible for the phenotypes resistant to the main antibiotics used in treatment.

The 399bp fragment of the *embB* gene related to ethambutol resistance could be amplified from five (5) positive samples (**Figure 1A**). Regarding resistance to isoniazide, the 457bp fragment of the *inhA* gene, the 703bp fragment of the *katG* gene, and the 587bp product of the *ahpC* gene could also be equally amplified from five (5) samples (**Figure 1B**).

Regarding resistance to rifampicin, the 349bp fragment of the *rpoB* gene could be amplified from ten (10) samples (**Figure 1C**).

The 940bp hotspot fragment of the gene responsible for pyrazinamide resistance, *pncA*, could be amplified from five (5) positive samples (**Figure 1D**).

Except for one sample, which presented a SSCP profile demonstrating mutation in the *embB* gene and that could lead to an
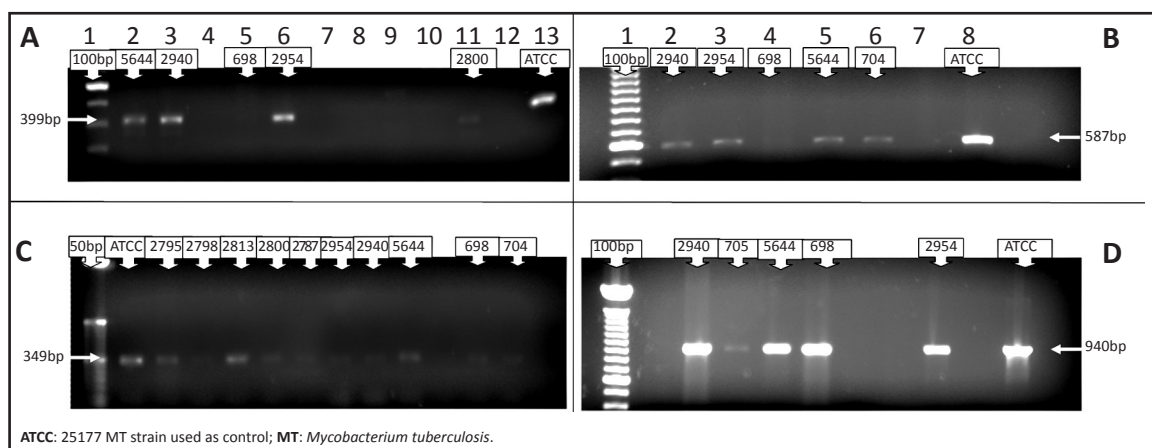
FIGURE 1 - Polymerase chain reaction amplification of hotspot mutation regions of the genes *embB* (A), *ahpC* (B), *rpoB* (C), and *pncA* (D), from samples with a positive result for MT diagnosis, either by culture, *IS6110* PCR, or bacilloscopy.

ethambutol resistant phenotype (**Figure 2**), all the samples from which it was possible to amplify a hotspot fragment of the genes responsible for *M. tuberculosis* resistance evaluated in this study presented a SSCP pattern similar to a native, nonresistant genotype, using the ATCC strain 25177 as control.
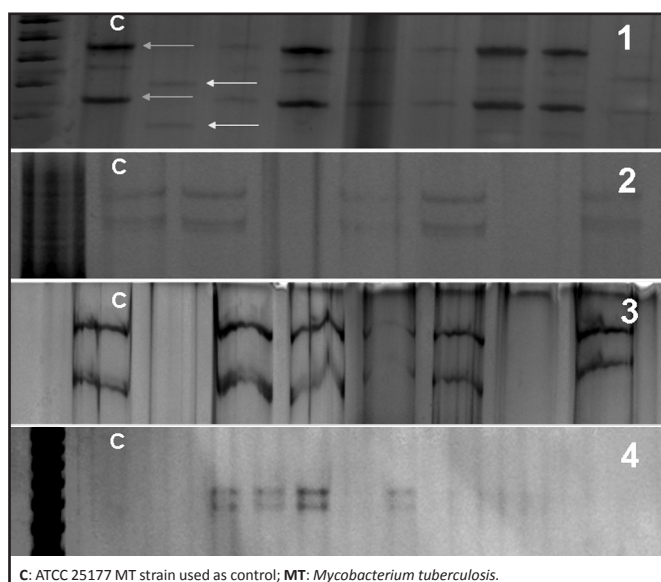

FIGURE 2 – Single-strand conformation polymorphism of polymerase chain reaction amplicons from the genes *embB* (1), *ahpC* (2), *rpoB* (3), and *pncA* (4), from samples with a positive result for MT diagnosis, either by culture, *IS6110* PCR, or bacilloscopy.

DISCUSSION

Estimates from the WHO indicate that nearly one third of the world's population is infected by the TB bacilli. About 8.8 million new cases are recorded every year, with 1.6 million deaths. Twenty-two countries harbor about 80% of all cases in the world, and Brazil occupies the 16th position in this ranking, with 70% of the cases concentrated in 315 of its 5,570 cities[12-14]. Strategies to stop the advance of this epidemic, however, are threatened not only by its synergic effect with HIV/AIDS but mainly by the increasing number of multiresistant strains. With the risk of the wide spread of resistant bacilli, the lack of stringent control and monitoring of patients may exponentially increase the rise of resistance, a drawback in the efforts on tuberculosis control[15,16].

A few reports point out a decrease in multiresistant TB cases in the last decade; however, most studies indicate the opposite. A study conducted in Brazil from 1996 to 1997 reported that 31% of analyzed strains were resistant to the main drugs used in treatment. *M. tuberculosis* presents a significant frequency of naturally resistant mutants: 1:106 for isoniazid, 1:108 for rifampicin, but less than 1:1014 for both simultaneously. Misuse of the drugs certainly contributes to the selection of resistant strains[13,17].

There are four known mechanisms through which bacterial resistance is acquired (conjugation, transformation, transduction, and mutation), but it is mainly by mutation that *M. tuberculosis* becomes resistant to drugs. Genetic and molecular analyses of resistant bacilli also suggest that resistance is acquired by mutations in the genes that codify the drug target. Unlike many other bacteria, MT does not perform horizontal gene transfer. The mechanism of multidrug resistance is still uncertain as no single gene involved has been identified; rather, it has been observed that multiresistant phenotypes arise from mutation in different genes[18].

Rifampicin binds to the beta subunit of RNA polymerase, which is codified by the *rpoB* gene, inhibiting the transcription process. Mutations at the *rpoB* produce conformational enzymatic changes and, therefore, resistance. About 95% of the rifampicin-resistant isolated MT strains have mutations in a 69bp region of this gene[13,19,20].

Several mutations, affecting one or more genes, have been found to be associated to isoniazid resistance; these include point mutations in genes such as the one that codifies the catalase-peroxidase enzyme (*katG*); the enoyl-ACP reductase, involved in the biosynthesis of mycolic acid (*inhA*); the alkyl hydroperoxide reductase, involved in cellular response to oxidative stress (*ahpC*), or, with lower frequency, even the β-ketoacyl-ACP synthase (*kasA*). Mutations in the *katG* and *inhA* genes are found in 75-85% of *M. tuberculosis* isolates resistant to this drug[20,21].

Pyrazinamide is a highly specific drug against *M. tuberculosis*, with no or minor effect against other *Mycobacterium* species but with a high level of intrinsic resistance observed. More than 70% of the *M. tuberculosis* strains resistant to pyrazinamide have mutations in the gene *pncA*, which codifies a pyrazinamidase enzyme that converts the drug to its active form. On the other hand, there is no perfect correlation between loss of pyrazinamidase activity and pyrazinamide resistance; mycobacteria may be resistant to the drug in spite of having enzyme activity[20,22].

The genetic bases of ethambutol resistance are associated with modifications in the *embB* gene in 70% of the resistant isolated strains. Mutations in multiple codons have been found, resulting in two, three, or four amino acid changes in the EmbB protein. The most frequent, however, were found in codon 306, a conserved methionine coding region[20,23].

In our study, we found no PCR-SSCP pattern that might correspond to a phenotype resistant to the main drugs used in MT treatment, except for one sample with a polymorphism in the *embB* gene that could correspond to resistance to ethambutol. For diagnostic purposes, that genotypic profile would have to be confirmed, ideally by sequencing; however, that is beyond the scope of this study. Our aim was to evaluate the PCR-SSCP technique as a screening tool for detection of *M. tuberculosis* resistance and, as a consequence, to obtain a notion of the prevalence of resistant strains in our population; the existing data lack scientific evidence, being only empirically suspected by the physicians in the public health system. The low prevalence of genotypic alterations is probably due to the efficiency and seriousness with which TB treatment is performed in our region. Tuberculosis drugs, as well as laboratory tests, are given at no cost to the patients, and medication is given through health agents, who personally deliver the drug to the house or workplace of the patient and then wait to see if the medicine is appropriately taken. Adhesion to treatment is very high. Only those patients who demonstrate commitment and capacity to take the medicine in the appropriate manner who earn the trust of the health agents are allowed to take home doses sufficient for a few weeks and take the drugs by themselves. However, surveillance and monitoring by the health agents continue to the end of the treatment period.

The failure of our procedure to detect some mutations must be taken into account. The detection of isoniazide-resistant strains, for instance, is quite complex, due to the number of genes involved and of regions to be analyzed. It is well known that the prevalence of the described mutations that may confer isoniazide resistance can vary according to the geographic location of the isolates; studies with *M. tuberculosis* strains in Brazil have demonstrated a significant variation, from 50 to 80%, depending on the region where the bacilli have been isolated. Mutations in the *katG* gene are responsible for 58 to 68% of the resistant strains, while 32% of the mutations appear in the *inhA* gene. Mutations in other sites, such as in the promoter region of the *ahpC* gene, are less frequent but do exist[10]. One must also consider that these apparently high rates of resistance actually do not represent the global prevalence of mutants in our population. In Brazil, requests for culture and antibiotic susceptibility testing in the public health system are only allowed for patients with conventional treatment failure or immunosupressed ones, which obviously produces a bias in these studies.

Another point to be considered in our study is that some mutations may not be detected because of limitations of the method. It should be noted that the sensitivity of the method is inversely proportional to the size of the amplified fragment. Thus, the number of mutations detected decreases when fragments smaller than 150 to 200bp are analyzed. Also, depending on the polyacrylamide concentration, the temperature, pH, or even the presence of additives, such as glycerol, the electrophoretic mobility, and the resolution may be affected[24].

It is also noteworthy that it was not possible to amplify the DNA of the studied genes from every sample with a positive result by culture. One reasonable explanation for this is the sensitivity difference between PCR with primers directed at the mentioned genes and PCR developed for diagnostic purposes. Even the amplification of the *IS6110* region of *M. tuberculosis* did not achieve the same sensitivity of culture for TB diagnosis. It is therefore expected that PCR designed to amplify regions of the genes where the mutations usually responsible for MT resistance are found would not be able to amplify the DNA of every positive sample.

Furthermore, as has already been demonstrated, in our population, samples from patients suspicious of having TB with a positive result by culture may actually be infected by non-*M. tuberculosis* species[7]. The genetic diversity among the *Mycobacterium* species sequences may prevent the studied genes from being amplified in PCR-SSCP.

It has been reported that the prevalence of resistant TB has significantly increased in the last years and, according to the Brazilian Society of Pneumology and Thisiology, it is possible that 50 million people around the world may be infected with resistant *M. tuberculosis*. Therefore, there is an obvious need for the availability of rapid and accurate tests capable of detecting resistant MT in a certain population. Automated culture methods with liquid media and antibiotic susceptibility testing have been validated and are accepted, and may reduce the wait for results to 10-40 days for culture and more than 10 days for drug sensitivity[5]; however, the cost of these methods largely supplant the value of the tests received by laboratories in Brazil. Polymerase chain reaction-single-strand conformation polymorphism may contribute to filling in this gap, with the increasing availability of laboratories capable of performing molecular diagnosis across the country.

According to the WHO, a prevalence of resistant bacilli higher than 2% has the inefficacy of the therapeutic scheme and the inefficiency of local TB control programs as principal causes. The prevalence of genotypic alterations indicated is only 0.01%, which certainly does not represent an additional problem in local control of the disease. In some parts of Brazil, a large proportion of patients fail to follow the adequate treatment regime[25]. In any case, it is obviously necessary to maintain surveillance for the purpose of monitoring and detecting an eventual rise in MT resistance rates in our population. In spite of its limitations, PCR-SSCP constitutes an easy, reliable, and fast method in this context.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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