Primary and acquired pyrazinamide resistance in patients with pulmonary tuberculosis treated at a referral hospital in the city of Recife, Brazil*

Resistência primária e adquirida à pirazinamida em pacientes com tuberculose pulmonar atendidos em um hospital de referência no Recife

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Abstract

Objective: To determine primary and acquired resistance to pyrazinamide in *Mycobacterium tuberculosis* strains isolated in sputum samples from patients with pulmonary tuberculosis. **Methods:** This was a prospective, descriptive study conducted between April and November of 2011 at a referral hospital for tuberculosis in the city of Recife, Brazil. Cultures, drug sensitivity tests, and tests of pyrazinamidase activity were conducted in a private laboratory in Recife. **Results:** Of the 71 patients included in the study, 37 were treatment-naïve and 34 represented cases of retreatment. Pyrazinamide-resistant strains were isolated in 14 (41.2%) of the 34 patients who had previously been treated for tuberculosis and in none of the 37 treatment-naïve patients. Of the 14 isolates, 10 (90.9%) tested negative for pyrazinamidase activity. A total of 60 isolates tested positive for pyrazinamidase activity. Of those, 56 (93.3%) were found to be sensitive to pyrazinamide. **Conclusions:** The high frequency of pyrazinamide-resistant strains (41.2%) in patients previously treated for tuberculosis highlights the need for drug susceptibility testing prior to the adoption of a new treatment regimen.

Keywords: Mycobacterium tuberculosis; Pyrazinamide; Tuberculosis, multidrug-resistant.

Resumo

Objetivo: Verificar a resistência primária e adquirida à pirazinamida em cepas de *Mycobacterium tuberculosis* provenientes de amostras de escarro de pacientes com tuberculose pulmonar. **Métodos:** Estudo prospectivo e descritivo realizado no período entre abril e novembro de 2011 em um hospital de referência para o tratamento de tuberculose em Recife (PE). Culturas, testes de sensibilidade a fármacos e testes da pirazinamidase foram realizados em um laboratório particular na mesma cidade. **Resultados:** Dos 71 pacientes incluídos no estudo, 37 eram virgens de tratamento e 34 eram casos de retratamento. Desses, 0 (0,0%) e 14 (41,2%), respectivamente, apresentaram cepas resistentes à pirazinamida. Desses 14 isolados, 10 (90,9%) apresentaram resultados negativos no teste da pirazinamidase. Dos 60 isolados que apresentaram resultados positivos para o teste da pirazinamidase, 56 (93,3%) eram sensíveis à pirazinamida. **Conclusões:** A elevada frequência de cepas resistentes à pirazinamida em pacientes em retratamento da tuberculose destaca a necessidade da realização de testes de sensibilidade à pirazinamida antes de se escolher um novo esquema de tratamento.

Descritores: Mycobacterium tuberculosis; Pirazinamida; Tuberculose resistente a múltiplos medicamentos.

Introduction

Multidrug-resistant *Mycobacterium tuberculosis* strains have emerged worldwide, and there is a need for rapid methods for diagnosis and determination of antituberculosis drug susceptibility.[1] There are few data on multidrug-resistant (MDR) tuberculosis and extensively drug resistant (XDR) tuberculosis in the 22 countries that, together, account for 80% of all tuberculosis cases worldwide, among which Brazil ranks 19th.[2]

In 2010 in Brazil, only 32% of the patients at risk of harboring drug-resistant *M. tuberculosis* strains (i.e., those who had regimen failure, had

* Study carrried out at the Otávio de Freitas Hospital, Recife, Brazil.

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recurrence, or dropped out of treatment and then returned) underwent drug susceptibility testing, and the northeastern region showed the lowest rate of testing (15.2%). In that year, 611 cases of MDR tuberculosis were reported, compared with 334 in 2001, i.e., there was an 82% increase between the years analyzed.[3]

Pyrazinamide is a first-line drug used for the treatment of tuberculosis in recently diagnosed patients and in patients with MDR tuberculosis.[4] Nevertheless, pyrazinamide susceptibility testing is not routinely performed in many laboratories, because of technical difficulties. Given that pyrazinamide requires an acid pH for being converted to its active form, i.e. pyrazinoic acid, by *M. tuberculosis* pyrazinamidase, there is a direct correlation between pH and the minimum inhibitory concentration of the drug. Therefore, a conflict occurs, especially because, at a lower pH, many mycobacterial strains are inhibited. Consequently, the variation in antibiogram methods affects the data on overall pyrazinamide resistance rates.[5,6]

In Brazil, most studies of antituberculosis drug resistance do not report any data on pyrazinamide susceptibility testing.[7,8] In addition, in the state of Pernambuco, for instance, there have not even been recent studies of antituberculosis drug resistance in general. The present study aims to fill this gap by reporting the rates of primary and acquired resistance to antituberculosis drugs, including pyrazinamide, in *M. tuberculosis* strains isolated in sputum samples from patients with pulmonary tuberculosis treated at a referral hospital in the state of Pernambuco.

Methods

This was a prospective, descriptive study conducted between April and November of 2011 at a referral hospital for tuberculosis in the state of Pernambuco, Brazil. Patients aged 18 years or older who had a positive sputum culture for *M. tuberculosis* were included. The study was approved by the research ethics committee of the hospital (Protocol no. 0.33.06.11).

A specific form was used to collect data on the following variables: age; gender; alcoholism; smoking; illicit drug use; cancer; diabetes; HIV serology; previous treatment with pyrazinamide; treatment dropout or irregular medication use; discharge as cured; and appropriate medication use.

The participants were divided into two groups. The first group, designated treatment-naïve (TN) group, consisted of recently diagnosed treatment-naïve patients treated at the hospital. The second group, designated retreatment (RT) group, included patients previously treated with first-line antituberculosis drugs, some of whom had received more than one course of treatment and some of whom had been hospitalized and treated with salvage drug regimens.

After sputum samples were collected and delivered to the laboratory, the specimens were processed with N-acetylcysteine-sodium-hydroxide.[9] Examination for AFB was performed by use of Ziehl-Neelsen staining. Culture for mycobacteria was performed on Löwenstein-Jensen solid medium and in Middlebrook 7H9 liquid medium enriched with oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson Co. Sparks, MD, USA). The improved medium was made selective by the addition of a mixture of antibiotics: polymyxin B; amphotericin B; nalidixic acid; trimethoprim; and azlocillin (PANTA; Becton Dickinson). After inoculation, the culture media were incubated at 37°C for 7 days, after which they were assessed for the presence of bacterial growth in order to detect any rapidly growing mycobacteria. Subsequently, they were reincubated and reassessed at 30 and 60 days, before the samples were classified as negative. Cultures were screened by real-time polymerase chain reaction (in-house method). The TaqMan platform was used, and the design of primers and probes was based on a previously validated protocol.[10]

All positive cultures underwent drug susceptibility testing. An indirect proportion method was used, as recommended by Canetti et al.[11] The drugs were added to the Löwenstein-Jensen medium at the following concentrations: streptomycin, 4 µg/mL; isoniazid, 0.2 µg/mL; rifampin, 40 µg/mL; and ethambutol, 4 µg/mL. The Löwenstein-Jensen media containing and not containing drugs were solidified by coagulation at 80°C for 45 minutes.

Regarding pyrazinamide susceptibility testing, a previous study[6] proposed 300 µg/mL pyrazinamide in 7H9-OADC liquid medium as the cut-off point for defining pyrazinamide resistance. Given that pyrazinamide is totally converted to pyrazinoic acid, pyrazinamide was theoretically estimated at 156 µg/mL, in accordance with the Henderson-

Hasselbalch equation. Therefore, in the present study, pyrazinamide susceptibility testing was performed in 7H9-OADC medium containing 200 µg/mL pyrazinamide, with pH being adjusted to 5.9, as recommended in that study.[6]

The pyrazinamidase activity assay was performed by the method of Singh et al.[1] For each case, two tubes were inoculated and incubated at 37°C. Four days later, 500 µg of a 1% ammonium ferrous sulfate solution, prepared immediately before use, were added to one of the tubes. Any color change from white to pinkish or brown was considered a positive reaction. If that tube tested negative, its duplicate was reincubated and reassessed at 10 days. Each culture testing negative for pyrazinamidase activity underwent a real-time polymerase chain reaction[12] for the purpose of potentially detecting a strain of *M. Bovis*, a species that is intrinsically resistant to pyrazinamide and, therefore, does not produce pyrazinamidase.

For data analysis, absolute and percentage distributions, as well as univariate and bivariate values, were obtained for categorical variables, and the statistical measures mean, median, and standard deviation were obtained for the variable "age" (descriptive statistical techniques). We used the Student's t-test with equal variances and either Pearson's chi-square test or Fisher's exact test when appropriate (inferential statistical techniques). To test the hypothesis of equality of means, we used Levene's F-test.

The margin of error used in deciding what statistical tests to use was 5.0%. Data were entered into an Excel spreadsheet, and statistical calculations were performed with the Statistical Package for the Social Sciences, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

We included 93 patients with suspected tuberculosis. Subsequently, 21 patients were excluded because they had negative sputum culture for *M. tuberculosis*, and 1 patient was excluded because he was infected with *M. bovis*, a strain that is intrinsically resistant to pyrazinamide. Therefore, the study population consisted of 71 patients.

The TN and RT groups comprised 37 (52.1%) and 34 (47.9%) patients, respectively.

Patient age in the TN group ranged from 21 to 76 years, with a mean of 41.0 years, a median of 40.0 years, and a standard deviation of 13.90 years. In the RT group, patient age ranged from 25 to 76 years, with a mean, a median, and a standard deviation of 40.00, 37.50, and 10.73 years, respectively. According to the Student's t-test with equal variances, there was no significant difference in mean age between the two groups ($p = 0.738$).

Table 1 shows the characteristics of the participants by group, with the following highlights: in the sample as a whole, most patients (67.6%) were male, slightly more than half (52.1%) were between 18 and 39 years of age, and alcohol and smoking were the most common comorbidities (in 71.8% and 59.2%, respectively), bearing in mind that the same patient could simultaneously have more than one comorbidity. In the RT group, all patients (100%) reported having previously been treated with pyrazinamide, 55.9% declared having dropped out of treatment, 20.6% reported having used the medication correctly, 14.7% reported having been discharged as cured, and the remaining 8.8% admitted having used the medication irregularly. For the chosen margin of error, alcoholism was the only variable for which there was a significant difference between the RT and TN groups (85.3% vs. 59.5%; $p = 0.016$).

Table 2 shows the susceptibility testing results of each of the five drugs tested. We can see that, in the TN group, there was one case of isoniazid resistance and one case of streptomycin resistance, whereas all of the other cases were classified as drug-susceptible. In contrast, in the RT group, the rates of drug-resistant cases ranged from 41.2% (pyrazinamide) to 85.3% (isoniazid), indicating significant differences between the two groups in the susceptibility testing results of all of the drugs tested ($p < 0.05$ for all).

Analysis of the association between the pyrazinamidase activity assay results and the pyrazinamide susceptibility testing results (Table 3) shows that the rate of pyrazinamide-resistant cases was higher in the absence than in the presence of pyrazinamidase (90.9% vs. 6.7%). This difference indicates a significant association between the presence of pyrazinamidase and pyrazinamide susceptibility ($p < 0.001$).

Discussion

This sample showed a predominance of male patients (67.6%), a result that is similar to those

Table 1 - Characteristics of the treatment-naïve and retreatment patients diagnosed with pulmonary tuberculosis who were treated at a referral hospital for tuberculosis in the city of Recife, Brazil, between April and November of 2011.

Variable	Group				Total		p
	TN		RT				
	n	%	n	%	n	%	
Gender							
Male	23	62.2	25	73.5	48	67.6	0.307*
Female	14	37.8	9	26.5	23	32.4	
Age, years							
18-39	17	45.9	20	58.8	37	52.1	0.519**
40-59	16	43.2	12	35.3	28	39.4	
60 or older	4	10.8	2	5.9	6	8.5	
Comorbidity							
Diabetes	6	16.2	1	2.9	7	9.9	0.109**
HIV	3	8.1	3	8.8	6	8.5	1.000**
No comorbidity	7	18.9	5	14.7	12	16.9	0.636*
Alcoholism	22	59.5	29	85.3	51	71.8	0.016*
Smoking	21	56.8	21	61.8	42	59.2	0.668*
Illicit drug use	8	21.6	7	20.6	15	21.1	0.915*
Tuberculosis retreatment							
Previous treatment with pyrazinamide			34	100.0			N/A
Dropout			19	55.9			N/A
Irregular medication use			3	8.8			N/A
Discharged as cured			5	14.7			N/A
Appropriate medication use			7	20.6			N/A
Total	37	100.0	34	100.0	71	100.0	

TN: treatment-naïve; and RT: retreatment. *Pearson's chi-square test. **Fisher's exact test.

Table 2 - Drug susceptibility testing results for the *M. tuberculosis* strains isolated from treatment-naïve and retreatment patients with pulmonary tuberculosis who were treated at a referral hospital for tuberculosis in the city of Recife, Brazil, between April and November of 2011.

Drug susceptibility testing	Group				Total		p*
	TN		RT				
	n	%	n	%	n	%	
Pyrazinamide							
Resistant	-	-	14	41.2	14	19.7	< 0.001
Susceptible	37	100.0	20	58.8	57	80.3	
Rifampin							
Resistant	-	-	22	64.7	22	31.0	< 0.001
Susceptible	37	100.0	12	35.3	49	69.0	
Isoniazid							
Resistant	1	2.7	29	85.3	30	42.3	< 0.001
Susceptible	36	97.3	5	14.7	41	57.7	
Ethambutol							
Resistant	-	-	19	55.9	19	26.8	< 0.001
Susceptible	37	100.0	15	44.1	52	73.2	
Streptomycin							
Resistant	1	2.7	17	50.0	18	25.4	< 0.001
Susceptible	36	97.3	17	50.0	53	74.6	
Total	37	100.0	34	100.0	71	100.0	

TN: treatment-naïve; and RT: retreatment. *Pearson's chi-square test.

Table 3 - Pyrazinamidase activity assay results and pyrazinamide susceptibility testing results for the *M. tuberculosis* strains isolated from patients treated at a referral hospital for tuberculosis in the city of Recife, Brazil, between April and November of 2011.

Pyrazinamidase activity assay	Pyrazinamide susceptibility testing				Total		p*
	Resistant		Susceptible				
	n	%	n	%	n	%	
Positive	4	6.7	56	93.3	60	100.0	< 0.001
Negative	10	90.9	1	9.1	11	100.0	
Total	14	19.7	57	80.3	71	100.0	

*Fisher's exact test.

reported in other studies.[13-17] With regard to age, the literature provides mixed findings. In this sample, tuberculosis primarily affected those between 18 and 39 years of age, a finding that was also reported in a survey conducted in Rio de Janeiro, Brazil.[14]

Comorbidities are risk factors for the occurrence of clinical presentations that are more severe and difficult to diagnose or that are responsible for affecting tuberculosis treatment success. Alcoholism and smoking are clearly related to MDR tuberculosis.[15,16] Other studies have revealed that alcoholism[18] and HIV/AIDS are factors associated with dropping out of pulmonary tuberculosis treatment.[19] In the present study, we found high rates of alcoholism (71.8%) and smoking (59.1%). This occurred similarly in the TN and RT groups, and it might be related to disease susceptibility. However, alcoholism was the only variable for which there was a significant difference between the TN and RT groups, and it might be associated with a higher rate of treatment dropout.

Tuberculosis/HIV co-infection was present at an equal rate in the TN and RT groups, demonstrating the close relationship between the two diseases. In fact, in developing countries, such as Brazil, tuberculosis is often the first opportunistic infection in HIV-infected individuals.[20,21] In a study conducted in the city of Belo Horizonte, Brazil, positive HIV serology was found in 12.5% of the tuberculosis cases.[22] In South Africa, where there is a high rate of HIV infection in the general population (over 20%) and low rates of cure with tuberculosis treatment, there has been an increase in the number of cases of MDR tuberculosis and numerous outbreaks of XDR tuberculosis, especially in hospitals and prisons where the tuberculosis infection control measures proposed by the World Health Organization in 1999 have not been adopted.[23]

In Brazil, most cases of MDR tuberculosis are cases of post-primary or acquired disease unrelated to HIV co-infection or institutional outbreaks and resulting from irregular medication use and treatment dropout. Among new tuberculosis cases, the rate of new cases of MDR tuberculosis is 0.9%.[21] In the present study, primary isoniazid resistance and primary streptomycin resistance, respectively, were found in 1 (2.7%) and 1 (2.7%) patient in the TN group. These rates are higher than the national average. However, the study was conducted at a referral hospital for tuberculosis, which might result in overestimation of rates. Nevertheless, there was no primary pyrazinamide resistance.

Pyrazinamide resistance was found in 14/71 strains. Of the patients from whom these strains were isolated, all (14/14) reported having previously been treated with this drug and 6/14 stated that they had dropped out of treatment. A study evaluating the drug resistance profile in a public referral center for tuberculosis in the city of João Pessoa, Brazil, showed that, of 22 patients, 12 (55%) were resistant to pyrazinamide, a rate that was higher than that found in our study, whereas 21 (95%) had previously been treated for tuberculosis.[15] Studies conducted in the states of Ceará and Minas Gerais, Brazil, found pyrazinamide resistance in 3.9% (59/1,500) and 6.38% (20/313), respectively.[17,24]

A survey conducted in South Africa reported that, of 127 drug-resistant *M. tuberculosis* strains isolated from previously treated patients, 68 (53.5%) were also resistant to pyrazinamide, and that only 1 of 47 *M. tuberculosis* strains (2.1%) were resistant to pyrazinamide alone, suggesting single-drug resistance.[5] In that same study, it was observed that, of the 68 pyrazinamide-

resistant strains, 62 (91%) were also resistant to isoniazid and rifampin.[5] In the present study, of the 14 pyrazinamide-resistant strains, 12 (85.7%) were also resistant to isoniazid and rifampin. Therefore, we can suggest that, in our hospital, pyrazinamide resistance is associated with resistance to other drugs, emphasizing the need for pyrazinamide susceptibility testing prior to treatment initiation, especially in patients previously treated for tuberculosis.

In a study conducted in Japan, pyrazinamide resistance was found in 53% of the MDR *M. tuberculosis* strains. All isolates testing positive for pyrazinamidase activity were found to be susceptible to pyrazinamide, whereas all isolates testing negative for pyrazinamidase activity were found to be resistant to pyrazinamide.[25] These data are consistent with those reported in a previous study.[26] In contrast, in a study conducted in Thailand,[27] pyrazinamide resistance was found in 6% (3/50) of the susceptible strains and in 49% (49/100) of the MDR tuberculosis strains. Pyrazinamidase activity was detected in 98 pyrazinamide-susceptible *M. tuberculosis* strains and in 18 of the pyrazinamide-resistant *M. tuberculosis* strains. Therefore, the pyrazinamidase activity assay had a sensitivity of 65.4% and a specificity of 100%. In that study conducted in Thailand, the sensitivity of the pyrazinamidase assay was low compared with those of the other methods used. Various factors, such as medium pH, inoculum size, growth stage, and the metabolic state of the bacillus, might have contributed to false resistance in the pyrazinamidase activity assay.[28] In the present study, 4 (28.6%) of the pyrazinamide-resistant strains were positive for pyrazinamidase activity, and 1 strain (1.7%) was negative for pyrazinamidase activity, although it was susceptible to pyrazinamide. This suggests that other genomic regions, different from the *pncA* gene, govern pyrazinamide resistance with the support of alternative mechanisms of resistance, such as insufficient drug uptake or the presence of an active efflux pump, which would limit the usefulness of the pyrazinamidase activity assay.

Previous studies have also reported that pyrazinamide-resistant *M. tuberculosis* strains were positive for pyrazinamidase activity.[1,28] Singh et al. found that, of 35 pyrazinamide-resistant strains, 6 were positive for pyrazinamidase activity. It should be highlighted that all strains were isolated from patients who had previously been treated for tuberculosis,[1] similarly to those used in the present study. The pyrazinamidase activity assay, because of its simplicity and low cost, might be important as a screening method for evaluation of pyrazinamide resistance, especially in developing countries, although its sensitivity and specificity must be considered in the analysis of results.

The rate of pyrazinamide-resistant strains (41.2%) found in the tuberculosis patients in the RT group in the present study highlights the need for pyrazinamide susceptibility testing in the follow-up of these patients.

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