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Molecular characterization of pre-extensive drug resistant *Mycobacterium tuberculosis* in Northeast Brazil

Thales Alves Campelo [1], Luana Nepomuceno Costa Lima [2,3], Karla Valéria Batista Lima [2,3], Caroliny Soares Silva [1], Marília Lima da Conceição [3], José Antonio Pereira Barreto [4], Aquiles Paulino Peres Mota [1], Soraya de Oliveira Sancho [1], Cristiane Cunha Frota [1]

ABSTRACT

In Fortaleza, the capital of Ceara State, Brazil, the detection rate of tuberculosis (TB) in 2018 was 65.5/100,000 inhabitants with a cure rate of 59.1%, which is higher than the country average. This study investigated the risk factors associated with drug-resistant tuberculosis (DR-TB) and identified the drug-resistance phenotype and resistance-conferring mutations. The geographic distribution of DR-TB in Fortaleza, Brazil, was also determined. From March 2017 to February 2018, 41 DR-TB isolates and 69 drug-susceptible pulmonary TB isolates were obtained from patients seen at a referral hospital in Fortaleza, Brazil. Samples were subjected to phenotypic and genetic analysis of resistance; the spatial distribution of the participants was also analyzed. Primary resistance was high (50.9%) among participants. The following risk factors for DR were identified: being female ($p = 0.03$), having diabetes ($p < 0.01$), history of previous TB disease ($p < 0.01$), and the number of intra-domiciliary contacts ($p < 0.01$). Analysis by multiplex allele-specific polymerase chain reaction detected mutations in the genes *katG* (65.8%), *rpoB* (43.9%), *inhA* promoter (14.6%), and *gyrA* (9.8%). Sequencing identified mutations in the the genes *katG* (75.6%), *inhA* promoter (19.5%), *rpoB* (85.4%), and *gyrA* (100%). There was no mutation in the *rrs* gene. Spatial analysis showed DR-TB isolates distributed in areas of low socioeconomic status in the city of Fortaleza. Our results emphasized the importance of detecting resistance to TB drugs. The resistance found in the gene *gyrA* is of concern due to the high number of pre-extensive DR-TB cases in Fortaleza.

KEYWORDS: *Mycobacterium tuberculosis*. Multidrug-resistant TB. Primary resistance. Pre-extensively drug-resistant TB. Risk factors.

INTRODUCTION

Brazil ranks 20th among the 30 countries responsible for 87% of new cases of tuberculosis (TB) globally[1]. In 2018, there were 72,788 new cases of TB with a case detection rate (CDR) of 34.8/100,000 inhabitants[2]. The World Health Organization (WHO) aims to reduce the TB CDR to less than 10.0/100,000[1]. Ceara State, located in the Northeastern region of the country, presented a CDR of 41.8/100,000 inhabitants, ranking 11th in the number of new cases among Brazilian states. In Ceara, new cases of TB mainly occur in Fortaleza, the State capital, including 61% of drug-resistant (DR)-TB cases, with an average cure rate of 70.3%, lower than the recommended rate of 85% worldwide[1,3]. Fortaleza is the fifth largest city in the country, administratively divided into six regions, with a population of approximately

[1]Universidade Federal do Ceará, Faculdade de Medicina, Departamento de Patologia e Medicina Legal, Fortaleza, Ceará, Brazil

[2]Instituto Evandro Chagas, Seção de Bacteriologia e Micologia, Ananindeua, Pará, Brazil

[3]Universidade do Estado do Pará, Programa de Pós-Graduação em Biologia Parasitária na Amazônia, Belém, Pará, Brazil

[4]Secretaria de Saúde do Município de Fortaleza, Fortaleza, Ceará, Brazil

Correspondence to: Cristiane Cunha Frota
Universidade Federal do Ceará, Faculdade de Medicina, Departamento de Patologia e Medicina Legal, Rua Monsenhor Furtado s/n, CEP 60441-750, Fortaleza, CE, Brazil
Tel: +55 85 33668640,
+55 85 99998-0805

E-mail: cristianefrota71@gmail.com

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2.6 million people[4]. In 2010, the Human Development Index of Fortaleza was 0.754, ranking 467th among the Brazilian municipalities[5].

In 2017, the Brazilian Ministry of Health developed a national plan to combat TB as a public health problem, aiming at eradicating the disease and reducing the incidence to less than 10.0/100,000 inhabitants by 2035[6]. Brazil has characteristics that favor the transmission of the disease, such as a great social inequality and a high rate of urbanization. Thus, in order to reduce the number of cases of TB, especially the transmission of primary resistant species, it is necessary to promote early diagnosis, identify socioeconomic and epidemiologic indicators, and identify the spatial distribution of clusters of cases. There are no reports on the characterization of resistance-related mutations against anti-TB drugs in Fortaleza, and also on the spatial distribution of cases of DR-TB.

Resistance to anti-*Mycobacterium tuberculosis* drugs is mainly caused by spontaneous mutations in drug-directed chromosomal genes or drug-modifying enzyme-related genes. Genetic studies have shown the presence of specific mutations in the genes responsible for conferring resistance to routine first-line and second-line anti-TB drugs. Mutations in *katG* and in the regulatory region of inhA (*inhA*-15) account for 42% to 95% and 6% to 43% of isoniazid (INH) resistance cases, respectively. For rifampicin (RMP), the most common mutation is in the resistance-determining region (RDR) of *rpoB*, with a 95% frequency, whereas for streptomycin (STR), most mutations (76%) occur in the 16S rRNA gene and the *rpsL* (52%-59%) genes. For quinolones, mutations are found in the genes *gyrA* and *gyrB* (75-94%). Resistance to ethambutol (EMB) is often associated with simultaneous resistance to INH, RMP and STR, and mutations in the *embCAB* gene[7].

Rapid diagnosis is required for DR-TB surveillance and control. Ideally, we should have a sensitive, rapid, and reasonably economical technique that does not require complex methods, as most cases of resistance occur in underdeveloped countries with limited economic resources. Thus, molecular techniques, such as allele-specific polymerase chain reaction (PCR) and sequencing, have been used in the detection of these mutations and to overcome the limitations of phenotypic testing[8].

The main objective of this study was to identify resistance-related mutations to the main first-line and second-line anti-TB drugs in Fortaleza and to compare phenotypic tests results (BACTEC Mycobacterial Growth Indicator Tube [MGIT] 960) with allele-specific PCR and sequencing. We have also investigated the risk factors associated with DR-TB and the geographic distribution of the study participants.

MATERIALS AND METHODS

This prospective case-control study was conducted from March 2017 to February 2018 in Fortaleza, Northeast Brazil. The study participants were recruited at the Messejana Hospital Dr. Carlos Alberto Studart Gomes, which is a reference center for the treatment of DR-TB. This is a 349-bed teaching hospital in Northeast Brazil, a leading hospital for treating patients with lung diseases, including DR-TB. On average, Messejana Hospital cares for 2,500 patients with DR-TB per year, and about 165 patients per month.

For the sample calculation, it was observed that 83 cases of multidrug-resistant (MDR)-TB were reported in 2013, 95 in 2014, 75 in 2015, and 45 cases in 2016. Regarding pre-extensively drug-resistant (XDR)-TB, 10 cases were reported in 2013, 21 in 2014, 5 in 2015, and 3 cases in 2016. The sample size for a finite population of at least 37 participants with DR-TB was estimated using a 95% confidence level ($\alpha = 0.05$), 50% of the population, and an error rate of 5%. The equation for the sample size calculation was $n = [N \times z^2 \times p \times (1 - p)]/[d^2 \times (N - 1) + z^2 \times p \times (1 - p)]$, where N is the total number of new smear-positive cases registered during one year at the hospital; the z value corresponds to the desired confidence level ($z = 1.95$); d is the absolute precision and p is the expected proportion of drug resistance in the target population[9]. The calculated sample size was increased by 20% to compensate for expected losses. Participants presented respiratory symptoms or chest radiography results suggesting pulmonary TB, confirmed by a clinical physician[10]. During the nursing appointment and after the laboratory confirmation of a new case of TB, participants were invited to take part in the study and to answer the questionnaire, which included demographic, socioeconomic, clinical, and behavioral data.

Patients with pulmonary TB who signed the informed consent and were at least 16 years old were included in the study. In the case of participants under the age of 18, parental or legal guardian consent was also obtained. The inclusion criterion of cases was resistance to at least one anti-TB drug confirmed by mycobacterial culture. Control patients were those with TB susceptible to treatment, as confirmed by a negative laboratory result according to the national TB program guidelines. The final sample size was 110 (41 cases and 69 controls). Patients infected with HIV, presenting extra-pulmonary TB and those without a fixed residence were excluded from the study.

This project was approved by the Ethics Committee of the Federal University of Ceara, Fortaleza, Ceara State, Brazil (protocol Nº 1.956.894). All participants (and/or legal

guardians) signed an informed consent form and authorized the sample collections.

Sputum samples were subjected to microscopic examination and culture in Löwenstein-Jensen medium. *M. tuberculosis* isolates were tested for susceptibility to first-line anti-TB drugs (STR, INH, RMP, and EMB) in an automated system (BACTEC MGIT 960)[11]. The critical concentrations of drugs in the medium were as follows: STR 1 µg/mL, INH 0.1 µg/mL, RMP 1 µg/mL, and EMB 5 µg/mL according to WHO guidelines[9]. Samples were incubated until they became positive or for a maximum of 42 days to deliver a negative result. For DNA extraction, samples considered positive were removed from the equipment, and sub-cultured in Löwenstein-Jensen medium. The sub-cultures were incubated for up to 20 days at 37 °C to evaluate possible contaminants. DNA extraction was performed as described earlier[12].

The multiplex allele-specific (MAS)-PCR protocol and primer sequences for the *katG315*, *inhA*-15 (*inhA* promoter), *rrs* A1401G, *rpoB* codons 516, 526 and 531 as well as *gyrA* D94G were used as described previously[13-16]. Each assay, for each of the genes involved three primers: an external forward primer, an external reverse primer and an allele-specific internal reverse primer, with the exception of the protocol described for the simultaneous analysis of *rpoB* codons 516, 526 and 531, which used a set of four primers. All allele-specific primers target the reference strain *M. tuberculosis* H37Rv allele. After amplification, PCR products were electrophoresed in 3% agarose gels in Tris-borate EDTA buffer at 120 V for 40 min, and visualized under ultraviolet light.

The oligonucleotides for Sanger sequencing were designed by the authors using the Primer3Plus program based on the genomic region of *M. tuberculosis* H37Rv, deposited in GenBank (NCBI: txid83332)[17]. Table 1 shows the primer sequences used for amplification in each of the five sequencing assays. PCR products were purified using a BigDye Xterminator Purification Kit (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) before sequencing in an Applied Biosystems DNA Sequencer. Sequences were analyzed using SecScape software v.2.7 (Applied Biosystems) and the BioEdit Sequence Alignment Editor version 7.2.5 (BioEdit, Carlsbad, CA, USA). *M. tuberculosis* strain H37Rv sequence was used as to prototype to align and analyze the obtained sequences.

The addresses of all the participants were geo-referenced using MyGeoPosition.com[18]. The coordinates were entered into a Microsoft Excel 2013 datasheet and transferred to the ArcGIS 9.3 program (Environmental Systems Research Institute, Redlands, CA, USA) for mapping and illustration of case contact networks. The ArcMap application provided information on maps showing the distribution of geo-referenced cases in Fortaleza according to drug resistance. We used a search radius of 10 km and grid size of 2.5 km.

A bivariate analysis was performed for all the variables of interest, for the cases and controls. SSPS v10.0 and Epi-info v6.04d were used for analyses. The chi-squared test was used to compare the proportions and differences, which were considered significant at $p \leq 0.05$. The Student's t test was used to compare the averages of continuous variables. The Mann-Whitney U test was used to compare data with high asymmetry.

RESULTS

The average age of the participants in this study was 40.5 years (the participants in the control group were younger), and almost half of the participants had not been previously treated for TB (56/110 or 50.9%). Table 2 presents a descriptive statistical analysis of the main characteristics

Table 1 - Primer sequences used in individual sequencing assays, melting temperature and molecular weight of products.

Primers	Sequence (5´- 3´)	Tm (°C)	Molecular weight (bp)
katG315F	GCTTCAAGACGTTCGGGTTC	59.5	840
katG315R	GGCAATCTCGGCTTCGC	60.8	
-15inhAF	CGGGAAGATCCGCGTCG	60.3	806
-15inhAR	CTGCCGGAGACCGAACCT	61.3	
rpoBF	GCGYCGGYCGCTATAAGGTC	60.7	818
rpoBR	CGAGACGTCCATGTAGTCCA	58.9	
gyrAF	CGCAACCCTGCGTTCGAATTG	62.8	809
gyrAR	CCTCAACAACRCCGCGCAT	61	
rrsF	ATAGGCGTTCCCTTGTGGC	60.1	778
rrsR	CACAAGAACACGCCACCG	60	

bp- base-pair

Table 2 - Demographic and clinical characteristics of the study participants in the city of Fortaleza, from March 2017 to February 2018.

Variables	Total N = 110 (%)	Cases N = 41 (%)	Controls N = 69 (%)	p[a]	OR (95% CI)
Age (years)					
Mean (SD)	40.5 (13.4)	41 (11.2)	40 (14.7)		
< 39	48 (43.6)	17 (41.5)	31 (44.9)	1	
40 - 49	40 (36.4)	15 (36.6)	25 (36.2)	0.84[c]	0.91 (0.4-2.2)
> 50	22 (20)	9 (21.9)	13 (18.8)	0.66[d]	0.79 (0.3-2.2)
Gender					
Male	68 (61.8)	20 (48.8)	48 (69.6)	**0.03**	0.4 (0.2-0.9)
Female	42 (38.2)	21 (51.2)	21 (30.4)		1.0
Ocuppation					
Unemployed	79 (71.8)	32 (78.1)	47 (68.1)	0.26	1.7 (0.7-4.1)
Employed	31 (28.2)	9 (21.9)	22 (31.9)		1.0
Diabetes					
Yes	20 (18.2)	15 (36.6)	5 (7.2)	**<0.01[b]**	7.4 (2.4-22.4)
No	90 (81.8)	26 (63.4)	64 (92.8)		1
Alcohol use					
Yes	63 (57.3)	24 (58.5)	39 (56.5)	0.84	1.1 (0.5-2.4)
No	47 (42.7)	17 (41.5)	30 (43.5)		1
History of previous disease					
Yes	54 (49.1)	31 (75.6)	23 (33.3)	**<0.01[b]**	6.2 (2.6-14.8)
No	56 (50.9)	10 (24.4)	46 (66.7)		1
Number of intradomiciliar contacts					
≤ 3	69 (62.7)	19 (46.3)	50 (72.5)	**<0.01**	1
≥ 4	41 (37.3)	22 (53.7)	19 (27.5)		3.1 (1.4-6.8)

Cases: drug-resistant tuberculosis; Controls: drug-susceptible tuberculosis; SD, standard deviation; OR, odds ratio; 95% CI, 95% confidence interval. [a]Pearson's chi square test; [b]Fisher's exact test; [c]Comparison of 40 to 49 years old and over 50 years old participants; [d]Comparison of under 39 years and over 50 years participants.

of the participants. Diabetes ($p < 0.01$; odds ratio [OR], 7.4; 95% confidence interval [CI], 2.4-22.4), history of previous disease ($p < 0.01$; OR, 6.2; 95% CI, 2.6-14.8), and a high number of intra-domiciliary contacts ($p < 0.01$; OR, 3.1; 95% CI, 1.4-6.8) were the most frequent variables in the case group. Female gender was also more frequent among the cases (51.2%; $p = 0.03$). There were no differences between cases and controls considering age, occupation, schooling, alcohol consumption, use of illicit drugs, psychiatric disorder and chronic obstructive pulmonary disease.

From the total of 41 isolates included in the study, 12 (29.3%) were resistant to only one drug, 14 (34.1%) were resistant to two drugs and 15 (36.6%) were resistant to three or more drugs. Simultaneous resistance to RMP and INH and to all first-line tested drugs (RMP, INH, EMB, and STR) were the most frequent (26.8% and 24.4%, respectively).

The frequencies of mutations detected by sequencing of genes related to INH, RMP, STR, and fluoroquinolone (FQ) resistance are shown in Table 3. In relation to INH, eight C→T mutations (21.6%, 8/37) were found at position 15 of the *inhA* promoter region in the resistant profile samples in the BACTEC system, and six were positive in MAS-PCR. Regarding the gene *katG*, the presence of an S315T mutation was found in 31 isolates (75.6%). Three isolates with an S315T mutation presented a susceptible profile in BACTEC system, and 27 of the isolates with the same mutation were positive in MAS-PCR.

Mutations in the RDR were found in 35 isolates, of which 4 (9.7%) had a susceptible profile in the BACTEC system and 18 (43.9%) were positive in MAS-PCR. The most frequent mutation was at codon 531 in 17 isolates (48.9%), followed by codon 516 in 15 isolates (42.8%) with mutations at gAc→gTc (53.4%) and Gac→Tac (46.6%). Mutations in codon 531 were found in two isolates with susceptible phenotypes.

Table 3 - Mutations observed in *inhA*-15, *rpoB*, *katG*, *rrs* and *gyrA* genes, phenotypic resistance and MAS-PCR positivity in 41 resistant isolates of *M. tuberculosis.*

Drug	Mutation by gene location	N (%)					
		Total of Resistant samples	Total of Susceptible samples	Total of mutated samples [a]	Resistant with mutation	Susceptible with mutation	MAS-PCR[a]
INH		37 (90.1)	4 (9.9)				
	mabA						
	-15 position (C/T)			8 (19.5%)	8 (21.6%)	0	6 (14.6)
	katG						
	codon 315						
	S315T (aGc/aCc)			31 (75.6)	28 (75.7)	3 (75)	27 (65.8)
RMP		37 (90.6)	4 (9.7)				
	rpoB			35 (85.4)			18 (43.9)
	codon 516			15 (42.8)	15 (100)	0	15 (83.3)
	D516Y (Gac/Tac)			7 (46.6)	7 (100)	0	
	D516V (gAc/gTc)			8 (53.4)	8 (100)	0	
	codon 526			3 (8.3)	3 (100)	0	3 (16.7)
	H526Y (Cac/Tac)			1 (33.3)	1 (100)	0	
	H526R (cAc/cGc)			2 (66.7)	2 (100)	0	
	codon 531			17 (48.9)	15 (88.2)	2 (11.8)	0
	S531L (tCg/tTg)			17 (100)	15 (100)	2 (11.8)	
AG		18 (43.9)	23 (56.1)				
	rrs						
	codon 1401			0	0	0	0
FQ		nt	nt				
	gyrA			41 (100)[a]			4 (9.8)
	codon 89			1 (2.4)			0
	D89A (gAc/gGg)			1 (2.4)			
	codon 90			10 (24.4)			0
	A90E (gCg/gAg)			2 (4.9)			
	A90V (gCg/gTg)			8 (19.5)			
	codon 94			6 (14.6)			4 (100)
	D94G (gAc/gGc)			5 (12.2)			
	D94N (Gac/Aac)			1 (2.4)			
	codon 95			29 (70.7)			0
	S95T (aGc/aCc)			29 (70.7)			

[a]Total of isolates was used as reference. S: susceptible; R: resistant; MAS-PCR: multiplex allele specific-polymerase chain reaction; nt: not tested; INH: isoniazid; RMP: rifampicin; AG: aminoglicosyde; FQ: fluoroquinolona. Amino acids codes: A: alanine; D: aspartic acid; E: glutamic acid; F: phenylalanine; G: glycine; H: histidine; L: leucine; N: asparagine; R: arginine; S: serine; T: threonine; V: valine; Y: tyrosine.

No mutations were found at positions 1009 and 1787 of the *rrs* gene, and no mutations were detected in the samples analyzed by MAS-PCR. However, 43.9% (18/41) of these isolates were resistant to STR in the BACTEC system. Regarding FQ, mutations associated with a resistant phenotype were found in all the samples. Mutations were detected at codons 89 (D89A), 90 (A90E and A90V), 94 (D94G and D94N) and 95 (S95T). Mutations in codon 95 were the most frequent (29/41; 70.7%), followed by codon 90 (10/41; 24.4%), codon 94 (6/41; 14.6%) and codon 89 (1/41; 2.4%). MAS-PCR was positive in four of the six isolates with mutation in codon 94.

A total of 41 samples were analyzed for RMP and INH resistance in the BACTEC system and MAS-PCR (Table 4). Twenty-eight isolates (75.6%) showed resistance to RMP and were positive in MAS-PCR ($p < 0.03$). Of 41 samples subjected to *rpoB* gene sequencing, 94.3% (33/35) were resistant according to the BACTEC system and were positive by sequencing ($p = 1.0$). In relation to INH, we verified that 97.3% (36/37) of the clinical isolates presented resistance in the BACTEC system and were positive in MAS-PCR ($p < 0.56$) corresponding to a sensitivity of 97.3%. In addition, we verified that 88.9% (32/36) of the samples were positive in the BACTEC system and by sequencing ($p = 0.71$).

Forty-three of the isolates were from participants living in Fortaleza city; the remaining (n = 6) were from the countryside of Ceara State. The spatial analysis (Figure 1) showed that DR-TB cases were spread throughout the city, predominantly in the Western region. Most of the cases were located within administrative regions 1, 5, and 6, with 11, 10, and 9 participants with DR-TB, respectively. The other administrative regions (2, 3, and 4) had a total of eight DR-TB cases.

DISCUSSION

This study showed that most of the drug-resistant participants have not been previously treated (75.6%), and all the isolates presented mutation in the *gyrA* gene. The frequency of primary resistance was high in relation to that found in Brazil, in Ethiopia and Serbia[19-21] and lower than the one found in Bangladesh[22]. The cases of DR-TB were spread throughout the city, raising concern about the increase in the number of cases bearing primary resistant *M. tuberculosis*.

Regarding the number of intra-domiciliary contacts, drug resistance was more frequent among those who reported having four or more intra-domiciliary contacts. It has already been reported in Amazonas State, Brazil, that DR-TB patients have an average of five family contacts[23]. However, a cohort study conducted in 2015 in Lima, Peru, found that crowding was associated with a greater incidence of secondary cases of TB disease among household contacts of drug-susceptible TB patients[24]. We have found that women are at a higher risk of resistance than men. A study conducted in Sokobanja, Serbia, found no differences in gender between TB-susceptible and DR-TB[21], whereas others found that males were at higher risk[25,26].

We found that diabetes was a comorbidity associated with resistance to anti-TB drugs. Data point out to the impact of diabetes on the immune system, favoring the bacillus survival[27]. Diabetes causes increased susceptibility to TB through several mechanisms, including hyperglycemia and cellular insulinopenia, which have indirect effects on macrophage and lymphocyte function[27,28]. Diabetes is

Table 4 - Contingency analysis between BACTEC MGIT 960 positivity, MAS-PCR assay and genetic sequencing.

Detected mutation	BACTEC MGIT 960			p^a	kappa	s (%)
	Total	R	S			
	N (%)	N (%)	N (%)			
RMP						
MAS-PCR						
Yes	30 (73.2)	28 (75.6)	2 (50.0)	**0.03**	0.14	75.68
No	11 (26.8)	9 (24.4)	2 (50.0)			
Total	41 (100)	37 (100)	4 (100)			
Sequencing						
Yes	35 (85.4)	33 (94.3)	2 (33.3)	1	0.61	94.29
No	6 (14.6)	2 (5.7)	4 (66.7)			
Total	41 (100)	35 (100)	6 (100)			
INH						
MAS-PCR						
Yes	38 (92.7)	36 (97.3)	2 (50.0)	0.56	0.53	97.30
No	3 (7.2)	1 (2.7)	2 (50.0)			
Total	41 (100)	37 (100)	4 (100)			
Sequencing						
Yes	35 (85.4)	32 (88.9)	3 (60)	0.71	0.27	88.89
No	6 (14.6)	4 (11.1)	2 (40)			
Total	41 (100)	36 (100)	5 (100)			

RMP: rifampicin; INH: isoniazid; R: resistant; S: susceptible; s: sensitivity. [a]McNemar's Chi-Square test.

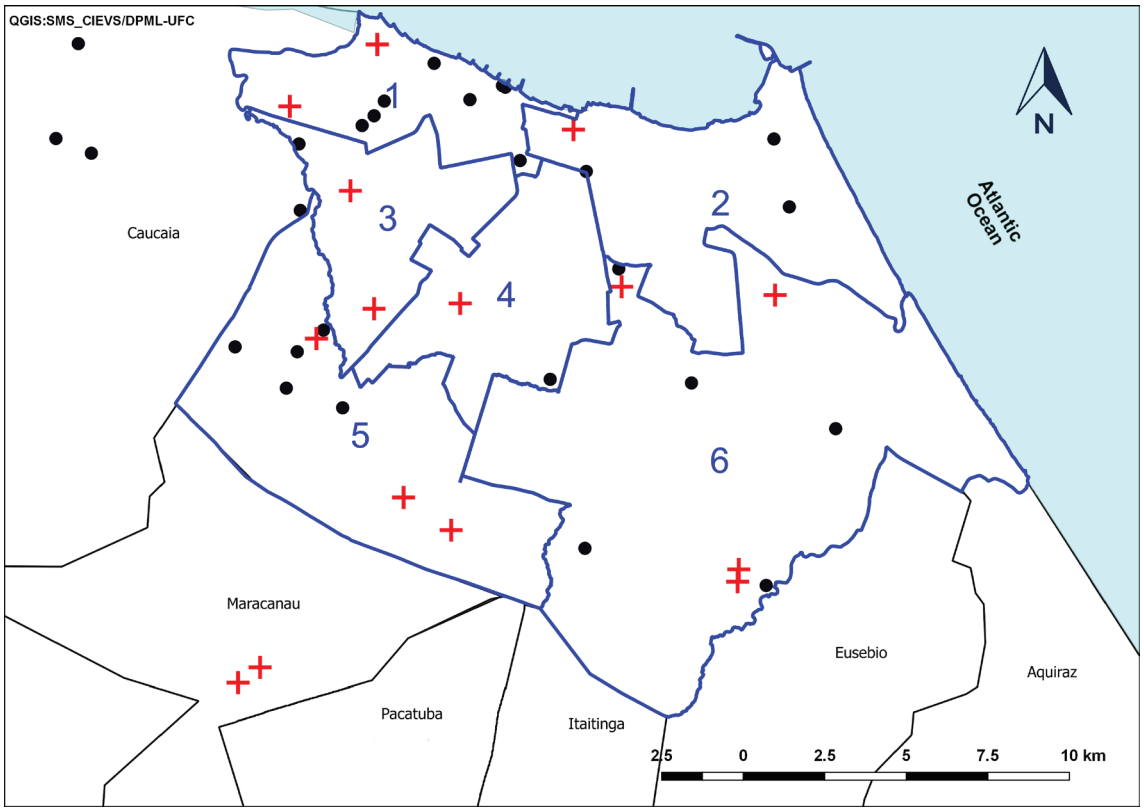

Figure 1 - Geographic distribution of the drug-resistant tuberculosis isolates in the city of Fortaleza, from March 2017 to February 2018. (+) resistance to ≥ 3 anti-TB drugs; (●) resistance to ≤ 2 anti-TB drugs.

associated with delays in *Mycobacterium* TB clearance during treatment, treatment failures, death, relapse and re-infection. Similar to other studies[22,29], we have found a 7.4 times higher risk of MDR-TB among diabetic patients with TB.

Mutations in the *gyrA* gene were also found in all the 41 samples, constituting the main mechanism of resistance to FQ, with specific mutations at codons 89, 90, 94 and 95. The identification of mutations in molecular markers of resistance characterizes these isolates as XDR-TB, with resistance to RMP, INH and at least one FQ drug or a second-line injectable drug[30]. Mutation at the S95T position of the *gyrA* gene was the most frequent mutation in our samples, followed by the A90V mutation. Other studies in China and the United States have reported the D94G codon mutation as the most common, ranging from 17.8% to 47.6%[31-33]. Mutations in A90E and S95T codons are not associated with resistance to FQs[33]. The S95T mutation has been described as a natural polymorphism (G284C). Identification of mutation in the *gyrA* gene is relevant for establishing FQ therapy, as specific mutations differ with respect to the minimal inhibitory concentration of ofloxacin and moxifloxacin[33].

Regarding aminoglycosides, no mutations were found at codon 1401 of the *rrs* gene with 43.9% of isolates resistant to STR in the BACTEC system. However, mutations have also been described in other genes, such as *rpsL* and *gidB*[7], and other genes related to efflux pumps[34].

Our sequencing results showed that the resistant phenotype for INH and RMP obtained by the BACTEC system could be accurately predicted. These findings are useful for estimating the sensitivity of sequencing, as well as of MAS-PCR, compared with the phenotypic test used in the BACTEC automated system, still considered the gold standard. However, as phenotypic tests of FQ resistance in Ceara State are not available, only the sensitivity values related to INH and RMP were calculated. Differences between phenotypic test results and molecular tests are explained by the presence of different mutations in uninvestigated genomic regions. Therefore, all discordant data were re-tested and sequencing data were reviewed. Other studies have also reported these disagreements between the phenotypic test (gold standard) and the molecular ones and have reinforced that phenotypic data should be interpreted cautiously in cases of therapeutic failure[8,35].

In our study, MAS-PCR had a sensitivity of 75.7% and 97.3% to detect mutations in isolates with resistance phenotype to RMP and INH, respectively. The approach is cost-effective and is a sensitive PCR-based technique to identify main mutations. However, it fails to detect

mutations outside the RDR in *rpoB* gene and in other genes related to drug resistance. Sensitivity can be maximized by exploring the other possible targets associated with resistance. Although sequencing is a costly and laborious process to confirm results, it can be used to diagnose drug-resistant isolates with high specificity and efficiency. Sequencing can also identify silent mutations that are not always related to phenotypic resistance.

Several limitations of this study should be considered in the interpretation of results. Firstly, only the Sanger sequencing platform was used, which has low genomic coverage. Secondly, the EMB-related gene (*embB*) and other alternative genes related to aminoglycoside, INH and FQ resistance were not investigated. As resistance to EMB is often associated with MDR and XDR resistance, these mutations were not investigated. In our study, 11 cases of DR-TB were resistant to EMB, and ten isolates had simultaneous resistance to INH, RIF and STR, and one isolate to INH. Thirdly, the number of patients studied was restrict to allow conclusive evidence regarding the sensitivity of the tests. In addition, in our study, the patients were recruited from only one hospital in Fortaleza that is a reference hospital for DR-TB treatment. Despite these limitations, the risk factors associated with DR-TB provide important information for health policy makers, and the molecular diagnosis has proved to be important for monitoring resistance in TB, especially in the detection of mutations considered as resistance markers.

Regarding the distribution of DR-TB cases, the concentration of participants domiciled in the Western part of city, in administrative regions 1, 5, and 6, is explained by the higher population density in these regions where poverty is common. Regions 1, 5, and 6 have the lowest average monthly income per person (from USD 121.98 to USD 178.8) in Fortaleza[36]. The richest neighborhoods in the city have average monthly incomes between USD 520.51 and USD 952.41. These poor regions in the city have also a high incidence of violence[37]. Fortaleza had an average incidence of 69.4 violent crimes per 100,000 inhabitants in 2015. The high rate of violent crime was associated with low economic development, limited population's access to infrastructure and health services[37]. Another study, also conducted in Fortaleza, reported a concentration of TB cases in insecure regions with low socioeconomic status[38]. Other studies conducted in Brazil have also associated a low Human Development Index with TB cases[39,40].

CONCLUSION

Our study showed a high prevalence of pre-XDR-TB in patients with no history of previous treatment in Fortaleza, presenting simultaneous resistance to various drugs. We have also shown the presence of pre-XDR-TB in geographic regions of the city that are associated with poverty and violence. We found that genetic sequencing is a useful tool for anti-TB drug-resistance surveillance, and molecular data should be routinely shared with public health agencies and used in conjunction with spatial analyses for the effective disease transmission control.

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AUTHORS' CONTRIBUTIONS

Cristiane Cunha Frota: conception, design, analysis and interpretation of data, article writing and critical review; Thales Alves Campelo: laboratory assays, analysis and interpretation of data, article writing and critical review; Caroliny Soares Silva, Aquiles Paulino Peres Mota and Soraya de Oliveira Sancho: laboratory assays, analysis and interpretation of data, article writing; Luana Nepomuceno Gondim Costa Lima, Marília Lima da Conceição and Karla Valéria Batista Lima: laboratory assays, analysis and interpretation of data, article writing and critical review; José Antonio Pereira Barreto: analysis and interpretation of data, article writing. All the authors read and approved the final version of the manuscript.

CONFLICT OF INTERESTS

The authors declare no conflict of interests.

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