Multi-Locus Sequence typing of Treponema pallidum in male patients with genital ulcers in a public STD clinic in South Brazil: a new alelle and almost complete macrolide resistance

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Multi-Locus sequence typing of *Treponema pallidum* in male patients with genital ulcers in a public STD clinic in South Brazil: a new allele and almost complete macrolide resistance.

Key Words: Genotyping, PCR, *Treponema pallidum*, syphilis, diagnostics

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Abstract

Objective: Considering the lack of data on *T. pallidum* genotyping in Brazil, we aimed to study its strains and their resistance to macrolides in genital ulcers suggestive of syphilis.

Methods: Men with genital ulcers suggestive of syphilis were invited to participate. Samples were collected with a dry cotton swab and immersed in a 0.9% NaCl solution. Detection was done by PCR amplification of 260 bp of the tpp47 gene. The PCR product was analyzed by electrophoresis in a 2% agarose gel containing 0.05% ethidium bromide. Positive PCR samples were analyzed by MLST (sequencing of chromosomal loci TP0136, TP0548, and TP0705). The A2058G and A2059G mutations in the 23S rRNA gene were evaluated by nested PCR. DNA sequencing was analyzed using Bioedit software (Tom Hall, USA). Genotyping was performed using the PubMLST online platform (Grillová scheme).

Results: All subjects were residents of Porto Alegre and aged between 19 and 66 years. Of the 43 samples, 32 were positive for PCR for T. pallidum. Thirty strains were available for genotyping and belonged to the SS14-like (73.3%) or Nichols-like (20%) Clonal Complex. Three complete MLST profiles were identified (1.3.1; 9.7.3 and 28.7.3), and a new allele was identified in one sample (approved by pubMLST curators as TP0705-22). Only one sample did not present the 2058 mutation in the 23S rRNA gene.

Conclusion: Our study identified genetic diversity in T. pallidum DNA using MLST with allelic variants for TP0136, TP0548, and TP0705, including a new allele. A single sample was characterized as genotypically susceptible to macrolides. All other samples (more than 95%) presented the A2058G mutation in the 23S rRNA gene, which causes resistance to macrolides. Improving understanding of the local epidemiology of T. pallidum with representative samples that allow cofactor analysis is crucial for prevention and care.

INTRODUCTION

Syphilis is a sexually transmitted infection (STI) of polymorphic evolution caused by the bacterium *Treponema pallidum* subspecies *pallidum.* The disease may occur asymptomatically, but, when presenting clinical signs, mucocutaneous lesions are frequent in the primary and secondary phases. Neurological and cardiovascular systems are the most affected, but any bodily system can be severely harmed leading to sequelae and even death. Transmission from the infected mother to the child can occur at any stage of pregnancy, leading to abortion, disease, or even death of the newborn. In addition to its direct consequences, the disease is a behavioral risk marker for HIV infection and other STIs. It is known that the presence of mucocutaneous lesions increases the transmission of HIV, either by the presence of inflammatory target cells or by the breakage of the mucocutaneous barrier.[1,2]

The World Health Organization (WHO), based on prevalence data from 2009 to 2016, estimated a total of 376.4 million curable STIs, of which 6.3 million were syphilis.[3] Although an increase in incidence is identified in high-income countries, the impact of the disease is much greater in middle and low-income countries. Although acquired syphilis belongs to a category of diseases that require compulsory reporting since 2010, underreporting of unknown magnitude is admitted. According to the Epidemiological Bulletin of Syphilis of the Ministry of Health of Brazil, published in 2021, the detection rate of acquired syphilis increased from 33.9 cases per 100,000 inhabitants in 2015 to 74,2 cases per

100,000 inhabitants in 2019. In the period from 2010 to 2018, the incidence rate of congenital syphilis increased from 2.4 to 8.5 cases per 1.000 live births.[4]

T. pallidum detection is difficult since it is non-cultivable with standard culture methods.[5] Diagnosis of syphilis is made by a combination of clinical and laboratory findings, which vary according to the stage of the disease.[4] Diagnostic methods are divided into direct and serological tests.[6] Among the direct ones are dark-field microscopy, direct immunofluorescence, and immunohistochemistry. In clinical practice, though, the diagnosis is presumptive. Serological treponemal tests (e.g., FTA-Abs) and non-treponemal tests (e.g. VDRL, RPR) are used. In the last decade, different molecular techniques of polymerase chain reaction (PCR) that detect *T. pallidum* genetic material have been used for diagnosis as well as for genotyping.[7,8,9] Moreover, DNA sequencing is increasingly used and allows studies of the genetic diversity, dynamics of transmission in different populations, virulence, and patterns of resistance to antibiotics.[8,10] More precisely, genotyping using the multilocus sequence typing (MLST) method on the chromosomal loci TP0136, TP0548, and TP0705 allowed better discrimination among *T. pallidum* strains from different regions of the world and was used to elaborate a database that allows epidemiological analyses (https://pubmlst.org/ organisms/treponema-pallidum).[11,12,13,14,15,16,17] Different loci characterize distinct alleles, and their combination defines the allelic profile and the sequence type (ST). A clonal complex gathers the strains of *T. pallidum* with the allelic or ST identical profiles.[18] In addition to the loci analyzed by MLST, an analysis of the 23S rRNA gene can complement the identification of mutations (A2058G or A2059G) which is associated to the resistance to macrolides.[19] Despite these advances,

standardized molecular tests are still scarce for the definitive diagnosis of active *T. pallidum* infection, and there is a lack of low-cost diagnostic tools with high sensitivities and specificities.[20]

To our best knowledge, Brazilian publications on molecular tests for syphilis are restricted to the diagnosis of *T. pallidum* infection and do not include genotypic studies. In the present study, exudate samples were obtained from genital ulcers suggestive of syphilis (primary or secondary) from male patients seen at a public STI clinic. The main objective was to increase knowledge about the local molecular epidemiology of *T. pallidum* circulating in Brazil.

MATERIALS AND METHODS

Men over the age of 18 years with genital ulcers considered to be manifestations of primary or secondary syphilis were invited to participate. They were being treated at the STD Unit of the Outpatient Clinic of Sanitary Dermatology (ADS) in the city of Porto Alegre, in South Brazil. The exudate samples of the lesions were collected between July 2019 and March 2020, using dry cotton swabs that were immersed in 0.9% sodium chloride solution, stored at 4ºC and sent to the Molecular Biology Laboratory of the Lutheran University of Brazil (ULBRA), where they were processed and analyzed. DNA extraction was realized by using the PureLink® Genomic Kit (Invitrogen®**,** Thermo Fisher Scientific, Carlsbad, California, USA) according to the manufacturer's recommendations using an aliquot of 200 µL of biological samples. After DNA release, samples were stored at -18 ºC until the next phase of our study. The detection of *T. pallidum* DNA was

performed by using the *primers* KO3 (5'-GAAGTTTGTCCCAGTTGCTGCTTT-3') and KO4 (5'-CAGAGCCATCAGCCCTTTTCA-3') which amplify a 260 bp segment of the *tpp47* gene as described previously.[21] Briefly, a mixture was prepared with a buffer 10X, 1.5 mM $MgCl_2$, 200 µM of each dNTPs, 25 pmol of each primer, and 1.25 Units of the enzyme *Taq platinum* DNA polymerase (Invitrogem, Thermo Fisher Scientific, Carlsbad, California, USA). Initial denaturation was performed at 95°C for 2 min, followed by denaturation at 95°C for 20 s, at 62°C for 20 s, and at 72°C for 20 s for 35 cycles, with a final extension at 72°C for 5 min. Amplification was performed using the Thermocycler MJ Research PTC 96 (Bioer Technology, Hangzhou, China). The PCR product was analyzed by electrophoresis in 2% agarose gel containing 0.05% ethidium bromide and visualized under ultraviolet light. At each PCR reaction, *T. pallidum* DNA obtained from samples known to be positive and ultrapure water were used as positive and negative controls, respectively.

The positive samples for *T. pallidum* by PCR were then analyzed for molecular typing utilizing the MLST method on the chromosomal loci TP0136, TP0548, and TP0705 as described previously[12]. The presence of the mutation A2058G and A2059G on the 23S rRNA gene responsible for macrolide resistance was evaluated by nested PCR. The first-round mixture consisted of 10X buffer; 2.0 mM MgCl2; 200 µM of each dNTPs; 1.2 µL primer at 10 pmol; and 1.25 U of *Taq platinum* DNA polymerase (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA) and 10 µL of biological samples. Initial denaturation was performed at 95°C for 5 min, followed by 30 s at 95°C, 30 s at 60°C, 2 min at 72°C for 35 cycles, and a final extension at 72°C for 7 min. In the second round of PCR,

5 µL of the amplified product was incorporated in a mixture with the same composition as the one used in the first round. Initial denaturation was performed at 95°C for 5 min, followed by denaturation for 30 s 95°C, 30 s at 62°C, 90 s at 72°C, for 35 cycles, and a final extension at 72°C for 7 min. All amplifications were performed on the MJ Research PTC 96 thermocycler (Waltham, Massachusetts, USA).

Sequencing of DNA was performed by ACTGene Molecular Analysis Inc (Porto Alegre, Brazil) using the final PCR products of each sample. The chromatograms were visualized and trimmed using Chromas software (Technelysium Pty Ltd), and the construction of contigs, alignment, and analysis of sequences was carried out using the Bioedit software (Tom Hall, Raleigh, North Carolina, USA). For genotyping analysis, we used the scheme including TP0136, TP0548, and TP0705 loci (MLST Grilová), and the platform public database for molecular typing and microbial genome diversity (PubMLST) available at https://pubmlst.org/organisms/treponema-pallidum. For resistance-related sequencing analysis, the reference sequence annotation corresponding to positions 2058 and 2059 in the rRNA gene of *Escherichia coli* (accession no. V00331) available at GenBank was used.

This project was approved by the Ethics Committee of the School of Public Health of Rio Grande do Sul under number 3,232,889, following Resolution 466 of the National Health Council (2012).

RESULTS

A total of 43 participants were recruited with ages ranging from 19 to 66 years old. All of them were residents of the metropolitan area of Porto Alegre, South Brazil. *T. pallidum*-specific sequence was detected in 32 (74.4%) of the 43 DNA samples analyzed. At least one locus of the MLST scheme was successfully sequenced in 30 of these 32 (93.7%), and the same proportion was obtained for 23S rRNA gene sequencing. Quality sequencing of TP0705, TP0136, and TP0548 was obtained in 22 samples. The combination of successfully sequenced loci among samples varied. Three allele variants were identified for TP0136, two for TP0548, and three for TP0705.

A new allele for TP0705 was identified in the only sample characterized as genotypic susceptible to macrolides, and it was called here allele 11, differing from allele 3 at loci position 270 (C270T). Three other samples presented heterozygous peaks (two peaks), at position 154 for TP0136, 14 for TP0548, and 265 for TP0705 (Figure 1). The results of *T. pallidum* typing, the identified alleles, the genotypic resistance profile, and the clonal complex are presented in Table 1.

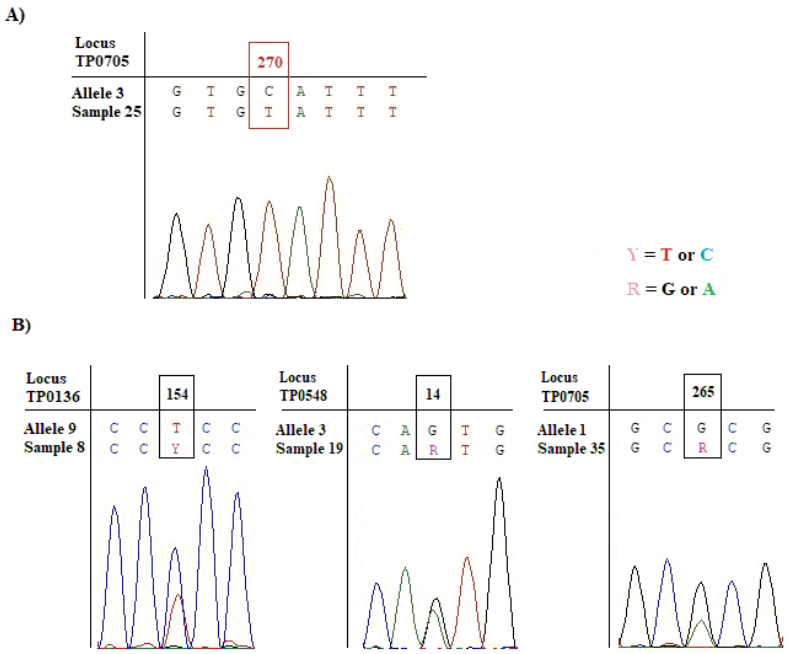

Figure 1: New allele for TP0705 and three heterozygous peaks for TP0136, TP0548 and TP0705 loci.

A) Chromatogram of sample 25 shown the new allele variant called here of 11, characterized by an SNV at position 270 (C→T) using the allele 3 (most similar sequence) as reference.

B) Chromatograms of three samples showed heterozygous peak for TP0136 locus at position 154 (C and T), TP0548 at position 14 (G and A) and TP0705 at position 265 (G and A). The alleles used as reference are the most similar sequence observed in PubMLST database.

Table 1: Genotyping of *T. pallidum* by Multi-Locus Sequence Typing (MLST) of studied samples.

Sample ID	TP0136	TP0548	TP0705	23S rRNA[1]	MLST[2]	ST[3]	Clonal Complex
1	1	3	1	R	1.3.1	1	SS14-like
7	nd	nd	1	R	x.x.1	-	SS14-like*
8	9‡	7		R	9.7.x	-	Nichols-like*
9	1	3	-	R	1.3.x	-	SS14-like*
10	1	nd	1	R	1.x.1	-	SS14-like*
11	1	3	1	R	1.3.1	1	SS14-like
12	1	3	nd	R	1.3.x		SS14-like*
13	nd	3	nd	R	x.3.x		SS14-like*
14	nd	nd	nd	R	-	-	nd
15	nd	nd	nd	R	-	-	nd
16	28	7	3	R	28.7.3	-	Nichols-like
17	nd	nd	1	R	x.x.1	-	SS14-like*
18	1	3	1	R	1.3.1	1	SS14-like
19	1	3‡	nd	R	1.3.x	-	SS14-like*
20	1	3	nd	R	1.3.x		SS14-like*
21	nd	7	3	R	x.7.3	-	Nichols-like*
22	9	7	3	R	9.7.3	26	Nichols-like
23	nd	nd	3	Nd	x.x.3	-	nd
25	nd	nd	11ǂ	S	x.x.11	-	nd
27	1	3	nd	R	1.3.x	-	SS14-like*
28	1	3	1	R	1.3.1	1	SS14-like
29	28	7	3	R	28.7.3	-	Nichols-like
30	1	3	1	R	1.3.1	1	SS14-like
31	nd	nd	1	R	x.x.1	-	SS14-like*
32	1	3	1	R	1.3.1	1	SS14-like
34	1	nd	1	Nd	1.x.1	-	SS14-like*
35	1	nd	1‡	R	1.x.1	-	SS14-like*
36	28	7	3	R	28.7.3	-	Nichols-like
38	1	3	nd	R	1.3.x	-	SS14-like*
39	1	3	1	R	1.3.1	1	SS14-like
40	1	3	1	R	1.3.1	1	SS14-like
42	nd	3	1	R	x.3.1	-	SS14-like*

[1] 23S rRNA gene encoding resistance to macrolide antibiotics: S = sensitive, R = resistant with mutation A2058G.

[2] Allelic profiles based on TP0136, TP0548 and TP0705 loci sequences.

[3] ST: Sequence Type. According to the PubMLST database for *Treponema pallidum subsp. pallidum*

* Clonal complex classified by approximation

ɬ New allele

ǂ alleles with heterozygous peak in one position of the sequence

nd = not determined

Sequence types (ST), which are attributed for fully characterized haplotypes (three MLST loci), were obtained from 11 samples, and the 1.3.1profile (ST 1) was the most frequent (8/11; 72,7%). The profile 28.7.3 has not yet been attributed a ST in the PubMLST platform. The samples that had at least one locus successfully sequenced were classified in a clonal complex by approximation; this classification was based in the number of sequenced isolates containing the alleles which appeared in the PubMLST database (supplementary Table 1). Two clonal complexes of strains were identified: 6/30 isolates (20%) were classified as Nichols-like and 22/30 (73.3%) as SS14-like. Two samples that had only the TP0705 locus successfully sequenced did not have a clonal complex determined.

Regarding resistance-related mutations, out of the 30 samples that were adequately characterized, only one did not have the A2058G mutation in the 23S rRNA gene, and therefore classified as susceptible. In two samples, a quality sequencing for this gene was not obtained.

DISCUSSION

Our study detected *T. pallidum* DNA in approximately two-thirds of the studied samples. Regarding profiles with unknown alleles, twelve allelic profiles were found. They belonged to the two best-known clonal complexes: SS14-like and Nichols-like. A new allele was identified in this study. It occurred in the two samples not classified as in a clonal complex (x.x.3 and x.x.11). The haplotype containing this allele had only the TP0705 locus characterized as allele 22 which was approved by the PubMLST curators (available at https://pubmlst.org/bigsdb?db=pubmlst_tpallidum_seqdef&page=alleleInfo&locus=TP0705&alle

le_id=22). It is known that this locus can share identical alleles among strains from SS14 and Nichols-clades, which makes it difficult to assign a clonal complex[12]. The three isolates presenting two peaks in some position of the allele sequence presented a partial allele match when analyzed by PubMLST. Some hypotheses arise to explain these heterozygous peaks in a haploid organism genome. As the different affinities of DNA polymerase for dideoxynucleotides (ddNTP), lead to differences in the rate of ddNTP incorporation and are also influenced by the upstream and downstream sequences - an effect known as “sequence context-dependent incorporation of dideoxynucleotide”, it may result in sequencing errors.[22,23,24] Also, contamination in sample manipulation, or even an intra-strain heterogeneity, may result in an ongoing adaptive diversification.[25] All but one of the identified DNA of *T. pallidum* presented the A2058G mutation in the 23S rRNA gene, which provides resistance to the macrolide class of antibiotics. The same clonal complexes identified in the samples of this study (Clonal Complex SS14 or the Nichols-like (20%) have been found in other studies, including Argentina, a country that borders our state.[12,16,17,27] Three MLST fully characterized profiles of *T. pallidum* were found (1.3.1, 9.7.3 and 28.7.3). The MLST profile 9.7.3 was also found in the Czech Republic and France.[17,30] This allelic profile corresponds to the Genotype U3U6 based on the SBMT that was also found in Argentina[26] and the subtype "g" ECDCT – TP0548[16], which was found in Switzerland, France, Italy, and the United Kingdom.[11,15,27,29,32] In turn, in Argentina, the most frequent subtype was the "f" ECDCT - TP0548.[26] The haplotype 28.7.3 which was not yet assigned an ST in the PubMLST database, was classified as Nichols-like complex clonal based on the genomic multi-centric collaborative study carried out by Beale et al..[3]

This profile was observed in isolates from Australia and United Kingdom between the years 2015 and 2019.

Although the ineffectiveness of azithromycin for the treatment of syphilis has been repeatedly demonstrated in clinical studies[1], it is important to mention that over 95% of the samples tested belong to *T. pallidum-resistant* strains. Our findings corroborate the ones of Grillová et al.[26,27] and Vrbova et al.[17] which demonstrated a high and increasing proportion of this mutation. Similarly, macrolide-resistance was identified in the United Kingdom[28], France[29], and Cuba.[30] On the other hand, Vaulet et al.[27] identified a much lower proportion of resistance in Argentina.

The usefulness of PCR for the diagnosis of primary and secondary syphilis, as well as the sensitivity and specificity of the method, have been extensively studied.[34] Facts that could explain the occurrence of negative samples: 1) some patients did not have active syphilis; 2) being self-limited manifestations, the number of microorganisms present in the lesions reduces over time; 3) the use of topical and systemic antibiotics before the consultation is also common.[6]

In addition, the diagnosis of syphilis is based on clinical aspects which have very low sensitivity and specificity. Positive serological results do not always mean an active infection, since these results might also indicate a treated past infection.[35] In addition, negative serological results can occur early in the course of the disease, even in the presence of primary lesions. These limitations of the current diagnostic tools, hence, justify improvement and standardization of molecular techniques. We believe that the in-house technique we developed and tested by the typing process in our study, may have paved the way for the development of affordable

PCR techniques which are in great need in settings where the incidence of syphilis is high which is the case in our country.

The small sample size may be considered a limitation of our study since it prevented the analysis of the associations of different genotypes with demographics, sexual practices, or geographic origin of participants. The SARS-COV2 epidemic hindered the enrollment of participants since it had a severe impact on health-seeking behavior. It reduced or even prevented patients from getting medical assistance in our clinic. Hence, the development of studies with larger sample sizes, or even the establishment of surveillance systems can provide valuable information for controlling syphilis.

active infection, since these results might also indicate a treated past infection.[35]

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CONTRIBUTORS

MCR, VMTG, LSE, MLRR, and MS were responsible for the conception and design. LSE carried out bioinformatic sequencing analysis and genotyping. MRCN participated in data collection and management. LTG carried out reference

organizing. All authors provided comments and edits and approved the final draft prior to submission.

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COMPETING INTERESTS

Competing interests. None declared.

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Supplementary table 1: Clonal complex of incomplete genotypic profiles based on number of samples present in PubMLST database.

Profile searched in the database	*n* of samples SS14-like	*n* of samples Nichols-like	*n* of samples without CC assignment	CC considered in this study
1.3.x	463	0	1	SS14-like
1.x.1	639	0	10	SS14-like
9.7.x	0	38	0	Nichols-like
x.3.1	477	0	4	SS14-like
x.3.x	482	0	6	SS14-like
x.7.3	0	41	3	Nichols-like
x.x.1	683	0	20	SS14-like
x.x.3	22	86	7	nd

CC = Clonal Complex

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