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Detection of *Treponema pallidum* in whole blood samples of patients with syphilis by the polymerase chain reaction

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ABSTRACT

Syphilis is caused by the bacterium *Treponema pallidum*. The diagnosis is based on clinical data and serological analysis; however, the sensitivity and specificity of such tests may vary depending on the type of test and stage of the infection. In order to overcome this premise, this study utilized the polymerase chain reaction (PCR) for the detection of *T. pallidum* DNA in whole blood samples of patients with syphilis. The blood samples from patients with or without symptoms of syphilis, but with positive results in enzyme-linked immunosorbent assay (ELISA), were included in this study. A venereal disease research laboratory (VDRL) test was performed for all collected sera samples. For PCR, the *T. pallidum* DNA was extracted from the collected blood samples and a specific primer set was designed to amplify 131 nucleotides of *polA* (Tp0105). The specificity of the primers was evaluated with the DNA of 17 different pathogens. From a total of 314 blood samples reactive in ELISA, 58.2% (183/314) of the samples were reactive in the VDRL test. In the PCR, 54% (168/314) of the ELISA-reactive samples were positive. In both tests (VDRL and PCR) 104 samples were positive. Of 104 positive samples for both tests, 71 were at the latent stage. Based on these results, it can be concluded that PCR with the designed set of primers can be utilized as a diagnostic method for *T. pallidum* detection in blood samples of patients with syphilis, especially those with latent infection. In addition, it can be utilized as a supplement for serological methods to improve the diagnosis of syphilis.

KEYWORDS: Syphilis. *polA* gene. *Treponema pallidum* DNA. Whole blood.

INTRODUCTION

Syphilis is a sexually transmitted infection caused by the spirochaete *Treponema pallidum* of the subspecies *pallidum* and results from direct contact with an infected lesion or by vertical transmission during pregnancy[1]. The incidence of syphilis has increased worldwide with an estimated 6.3 million new cases annually[2]. In Brazil, the number of syphilis cases steadily increased from 3,936 (2010) to 155,975 (2019) notified cases, according to a report by the Brazilian Ministry of Health[3]. Above all, a significant increase in cases among women has been reported, from 1,472 to 62,485 notified cases[3,4]. Based on the clinical data, syphilis is divided into three infectious stages (primary, secondary, and early latent) and two non-infectious stages (late latent and tertiary); these stages contribute to the orientation of the treatment and the follow-up of this infection[5].

Due to the absence of an optimal diagnostic test, the detection of syphilis has remained a challenge. This has led to the search for improving the diagnostic

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methods. Darkfield microscopy (DFM) is a direct detection method of *T. pallidum* in patients presenting lesions; however, it requires a trained laboratory team[6]. In the absence of lesions, serological tests for the detection of antibodies are used for the indirect diagnosis of syphilis. Treponemal tests are used to detect the specific antibodies for *T. pallidum* proteins, but they remain reactive for life in most patients[1]. Whereas, nontreponemal tests, such as the venereal disease research laboratory (VDRL) test, detect the antiphospholipid antibodies derived from damaged mammalian cells and treponemes as a result of the host immune response to the infection. The sensitivity and specificity of both tests vary according to the type of technique used, as well as the stage of syphilis[7]. False-negative results may occur, mainly in cases of primary syphilis, where serological tests exhibit low sensitivity and nontreponemal tests a sensitivity of 62–78%. Thus, multiple serological tests must be performed for the diagnosis of syphilis[8].

To diagnose the primary and secondary stages of syphilis, *T. pallidum* DNA detection by polymerase chain reaction (PCR) can be performed as a reference test. It is more precise than DFM, requires less expertise and has better reproducibility for use in routine diagnostics[9]. Previous studies have employed primers based on *T. pallidum* genes, such as *tpp47* (Tp0574) and *polA* (Tp0105)[10], for the detection of DNA by PCR in several samples, including whole blood, serum, plasma, cerebrospinal fluid, lesion exudate, fixed tissues, gastric lesions, urine, and intraocular fluid[11-15]. However, an optimal molecular marker for the diagnosis of syphilis is still not determined. In general, molecular tools provide an early and reliable diagnosis of syphilis, thereby assisting in preventing the transmission of this agent and contributing toward a reduced number of cases[16]. Moreover, the molecular amplification techniques can be used for *T. pallidum* strain typing from the clinical specimens. Therefore, it contributes to a better understanding of the pathogenesis and transmission of this pathogen[10]. In this study, we used PCR to amplify 131 nucleotides of *T. pallidum polA* (Tp0105) from whole blood samples of patients with or without symptoms of syphilis.

MATERIALS AND METHODS

Biological samples and serology testing

In this study, sera and whole blood samples of patients with or without symptoms of syphilis and with positive reaction in enzyme-linked immunosorbent assay (ELISA) for simultaneous detection of anti-*T. pallidum* IgM and IgG antibodies (ICE Syphilis, DiaSorin, Saluggia, Italy) were included[17]. Serial dilution of the sera samples for the titration of the VDRL test (Abbott Murex, Dartford, UK) was performed according to the manufacturer's instructions. The samples from patients without clinical symptoms, with no history of syphilis treatment, and reactive serology tests (ELISA and VDRL) were classified as latent syphilis. Whereas, the samples from patients with a typical chancre or ulcer and reactive serology tests (ELISA and VDRL) were classified as primary syphilis[18]. In addition, the syphilis stage was not determined for patients without clinical symptoms, with a history of syphilis treatment and with reactive serology tests (ELISA and VDRL).

DNA extraction of *T. pallidum*

The QIAamp DNA Blood Mini kit (Qiagen, Germantown, Maryland, USA) was used for the extraction of *T. pallidum* genomic DNA from 200 µL whole blood, according to the manufacturer's instructions. Subsequently, the DNA samples were quantified using the BioDrop µLITE equipment (BioDrop, Cambridge, UK), and the integrity of the extracted DNA was analyzed by electrophoresis on 1% agarose gel stained with GelRed 100X (Uniscience, Sao Paulo, Brazil). The DNA samples were stored at –20 °C for further analysis.

Construction of the synthetic gene

A 131-nucleotide fragment of *polA* (Table 1) was cloned into the pUC57-Amp vector (Genewiz, Nova Jersey, USA) and denoted pUC57/*polA_d*. After propagation in *Escherichia coli* TOP10 (Invitrogen, Carlsbad, CA, USA), the plasmid DNA was purified using the GenElute Plasmid Miniprep kit (Sigma-Aldrich, St. Louis, MO, USA) and used as a positive control in PCR.

PCR detection of *T. pallidum*

For the detection of *T. pallidum* DNA based on the PCR amplification of *polA*, primer sets were designed using Primer-BLAST[19] based on the sequence of the Seattle Nichols strain of *T. pallidum* (GenBank: CP010422.1) (Table 1). The PCR reaction volume of 25 µL contained 20–100 ng genomic DNA, 1x PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 0.5 µM of each primer (Sense: 5'-TGCGCACGAAGATAGTGTGT-3'; Anti-sense: 5'-AGCAGACGTTACATCGAGCGGA-3'), and 1 U of Taq DNA Polymerase (Invitrogen, Brazil). The reaction was subjected to amplification in a T100 (Bio-Rad, Hercules, CA, USA) thermocycler with the following cycling

Table 1 - Primers used for the PCR amplification and pUC57/*polA_d* vector.

Primer Pair	*polA* Sequence (5'-3')	Locus in Genome (CP010422.1)	Length
Forward	TGCGCACGAAGATAGTGTGT	116498-116517	131 bp
Reverse	AGCAGACGTTACATCGAGCGGA	116607-116628	

PolyAgene: A 50 T 76 C 48 G 51 | GC%: 44% | Length: 225
GGATCCAAGACGGCTGCACATCTTCTCCACTGTTTTGGCACACTTGATGGTATTTATCGTCATACCTATTCCTTAAAAGAAG
CGC**TGCGCACGAAGATAGTGTGT**GGGAAGAAAGATGCATTTTTTTCTCGTTCACTCATTGAGTTGCGTGACGATGTACCA
TGTGTTTTTTCGCTCGAAGATTCCTGTTGTAT**TCCGCTCGATGTAACGTCTGCT**TGAAAGCTT

conditions: initial denaturation at 94 °C for 5 min; 40 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min; and followed by a final extension at 72 °C for 5 min. For the positive and negative controls, pUC57/*polA_d* DNA and nuclease-free water (Sigma-Aldrich, USA), respectively, were used. The amplified product of *polA* was confirmed by sequencing (ABI 3730 xl DNA Analyzer, Applied Biosystems, USA). The human β-globin gene (268 base pairs)[20] was used as the internal control of the negative *polA* PCR reaction to assess the integrity and sufficiency of the extracted DNA.

PCR specificity test

The specificity of the primers was evaluated by PCR utilizing the DNA extracted from the following 17 pathogenic microorganisms: one yeast, three viruses, and 13 bacteria (Table 2).

Ethical approval

This study was approved by the Research Ethics Committee at the Federal University of Grande Dourados (Nº 191.877).

RESULTS

In this study, a total of 314 sera and blood samples from patients with positive serology in ELISA were included and subjected to VDRL and PCR assays. In total, 314 samples (sera and blood) from patients (236 men and 78 women) were included in this study. The median age of the patients

Table 2 - Microorganisms used for primers specificity assay.

Microorganisms	Type	Provenance
Leptospira interrogans	Spirochete bacteria	(CDTec/UFPel)[a]
Borrelia burgdorferi	Spirochete bacteria	
Neisseria meningitidis	Gram-negative bacteria	(LACEN/MS)[b]
Klebsiella pneumoniae	Gram-negative bacteria	(LPCS/UFGD)[c]
Acinetobacter baumannii	Gram-negative bacteria	
Serratia marcescens	Gram-negative bacteria	
Escherichia coli	Gram-negative bacteria	
Mycobacterium tuberculosis	Gram-positive bacteria	
Streptococcus pyogenes	Gram-positive bacteria	(LACEN/MS)[b]
Streptococcus pneumoniae	Gram-positive bacteria	
Streptococcus mitis	Gram-positive bacteria	
Streptococcus parasanguinis	Gram-positive bacteria	
Streptococcus oralis	Gram-positive bacteria	
Cryptococcus neoformans	Yeast	
Human papillomavirus (HPV)	Virus	(LPCS/UFGD)[c]
Human Immunodeficiency Virus (HIV)	Virus	(LPCS/UFGD)[c]
Hepatitis B Virus	Virus	

[a](CDTec/UFPel) - Centro de Desenvolvimento Tecnologico da Universidade Federal de Pelotas; [b](LPCS/UFGD) - Laboratorio de Pesquisa em Ciencias da Saude da Universidade Federal da Grande Dourados; [c](LACEN/MS) - Laboratorio Central de Saude Publica do Mato Grosso do Sul.

was 38 years (Range: 18–75) – the median for men was 38.1 and the median for women was 37.7 (Table 3). In the VDRL test, 58.2% (183/314) of the samples were reactive with titration ranging from 1/2 to 1/2,048 and 38.5% (121/314) samples were classified as latent syphilis, 3.8% (12/314) as primary and 15.9% (50/314) as undetermined syphilis, according to the guidelines of the Brazilian National Health System (SUS, acronym in Portuguese)[21] and the Centers for Disease Control and Prevention (CDC)[22]. Out of the 41.7% (131/314) negative samples in the VDRL test, 30.5% (96/314) were from patients who never tested for syphilis, 8.2% (26/314) with a history of syphilis, and 2.8% (9/314) with untreated syphilis.

Table 3 - Information about the patients for the results of VDRL and PCR assays.

	VDRL		PCR	
	Reactive	Non-reactive	Positive	Negative
Men	125	111	116	120
Women	58	20	52	26
Average age	36.5	40.2	38.2	37.8

The primer sets used in this study amplified a fragment of 131 bp from *T. pallidum*. In the PCR assay, 54% (168/314) of the samples were positive. All negative PCR samples for *polA* exhibited amplification for the human β-globin gene (Table 4). Analyzing these results with the stages of syphilis, 58.3% (7/12) of primary syphilis, 58.7% (71/121) of latent syphilis, and 52% (26/50) of undetermined stage syphilis samples were positive in PCR. An overview of the serology and PCR results is described in Figure 1.

Among the samples reactive in the ELISA test and non-reactive in the VDRL test (131/314), 48.8% (64/131) were positive in PCR, with 66.6% (6/9) of the samples being from the patients with untreated syphilis, 53.8% (14/26) from the patients with a history of syphilis, and 45.8% (44/96) from the patients who never tested for syphilis. The designed primers did not amplify the genetic material

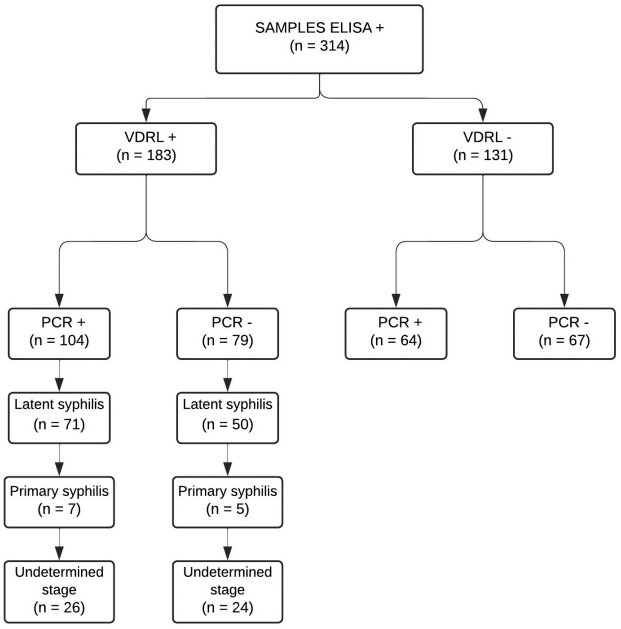

Figure 1 - Overview of serology and PCR results.

Table 4 - Comparative results among the samples reactive in VDRL and available in PCR.

VDRL Title	VDRL results		PCR results			
			Positive		Negative	
	n	%	n	%	n	%
1	6	1.91	6	100	0	0
1:2	47	14.97	20	42.55	27	57.45
1:4	38	12.10	23	60.53	15	39.47
1:8	34	10.83	19	55.88	15	44.12
1:16	16	5.10	9	56.25	7	43.75
1:32	6	1.91	6	100	0	0
1:64	18	5.73	10	55.56	8	44.44
1:128	8	2.55	7	87.50	1	12.50
1:256	3	0.96	2	66.67	1	33.33
1:512	2	0.64	0	0	2	100
1:1024	1	0.32	1	100	0	0
1:2048	4	1.27	3	75	1	25
Non-reactive	131	41.72	64	48.85	67	51.15
Total	314	100	170	54.14	144	45.86

from any of the evaluated microorganisms (Table 2) and, therefore, they were considered specific. The minimal DNA concentration possible to amplify by our PCR in positive control was 4×10^{-8} ng/μL.

DISCUSSION

Syphilis is considered a serious global public health challenge because of its difficult diagnosis, especially in patients without visible clinical manifestations. The untreated syphilis infection can lead to irreversible neurological or cardiovascular complications[23]. In this study, we used a novel set of primers for *T. pallidum* detection through PCR and compared PCR with serological techniques for *T. pallidum* detection. The PCR results from the proposed set of primers were in agreement with the results of the VDRL test in 56.8% (104/183) of the samples and demonstrated a positivity rate of 58% for latent syphilis in the positive whole blood samples.

Previous PCR-based studies targeting the *polA* demonstrated a detection rate of 6.2% (5/81)[24], 27.5% (19/69)[25], 61.5% (8/13)[26], and 61.9% (39/63)[27] in whole blood samples of patients with latent syphilis. In this stage, the lower number of *T. pallidum* cells/mL of blood compared to secondary syphilis is associated with greater dissemination of this bacterium[28]. Despite the disadvantages associated with whole blood, such as the presence of PCR inhibitors and low *T. pallidum* load, our results demonstrated the PCR ability to detect the pathogen DNA with high specificity even in asymptomatic patients or patients with latent syphilis. Vrbová *et al.*[29] demonstrated that the whole blood PCR positivity rate for the primary stage was 42.1% (40/95). In our study, the PCR positivity rate was similar: 58.3% (7/12) for primary syphilis. The recent detection of syphilis is important to reduce the transmission of the infection, but the early stages of syphilis are the most infectious.

The CDC describes the possibility wherein patients infected with *T. pallidum* can be reactive in treponemal tests and non-reactive in non-treponemal tests, indicating the use of reverse diagnosis[22]. In our study, 48.8% (64/131) of the samples reactive in ELISA and non-reactive in the VDRL test were positive in PCR, indicating the possibility of detection of *T. pallidum* in patients with early or late stages of the disease, with reduced amounts of antibodies to cardiolipin. The PCR positivity of whole blood samples correlated with the early stages of syphilis[29]. This result indicates that PCR can be employed as a confirmatory treponemal test in the reverse syphilis diagnostic protocols of SUS[21] and CDC[22].

The 51.1% (67/131) reactive ELISA samples negative in VDRL and PCR may be cases of treated syphilis, since the serological test detects the immunological memory (IgG)[30]. As reported in a previous study, the 22 patients with undetermined stages of syphilis, reactive ELISA and VDRL assays, and a negative PCR test, could be medicated patients without the presence of *T. pallidum* DNA in their bloodstream[31]. However, the false-positive results in serology exist due to the difficulty of decreasing antibody titers in some patients[1].

According to our results, all the negative *polA* PCR samples were positive for the β-globin gene, indicating the absence of PCR inhibitors in the extracted DNA. Furthermore, the primers designed in our study demonstrated specificity similar to those reported previously[32], which was indicated by the non-amplification of DNA samples from 17 other microorganisms, including *Leptospira interrogans*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus parasanguinis,* and *Borrelia burgdorferi.*

CONCLUSION

This study has limitations: for example, the evaluated samples were positive in ELISA; however, some could have been exposed and treated, thereby maintaining the immunological scar and leading to a selection bias. Furthermore, the sample size of this study was smaller, and samples of patients with secondary and tertiary syphilis were not included. Therefore, future studies based on a larger number of samples with different stages of syphilis are essential for better evaluation of this PCR technique. Overall, the results obtained in our study were consistent with the serological diagnosis of syphilis, indicating that the designed primer set may be a valuable, sensitive, and low-cost tool for screening and confirming the diagnosis of patients with latent infection.

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