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A retrospective cross-sectional quantitative molecular approach in biological samples from patients with syphilis

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ABSTRACT

Syphilis is the sexually transmitted disease caused by *Treponema pallidum*, a pathogen highly adapted to the human host. As a multistage disease, syphilis presents distinct clinical manifestations that pose different implications for diagnosis. Nevertheless, the inherent factors leading to diverse disease progressions are still unknown. We aimed to assess the association between treponemal loads and dissimilar disease outcomes, to better understand syphilis. We retrospectively analyzed 309 DNA samples distinct anatomic sites associated with particular syphilis manifestations. All samples had previously tested positive by a PCR-based diagnostic kit. An absolute quantitative real-time PCR procedure was used to precisely quantify the number of treponemal and human cells to determine *T. pallidum* loads in each sample. In general, lesion exudates presented the highest *T. pallidum* loads in contrast with blood-derived samples. Within the latter, a higher dispersion of *T. pallidum* quantities was observed for secondary syphilis. *T. pallidum* was detected in substantial amounts in 37 samples of seronegative individuals and in 13 cases considered as syphilis-treated. No association was found between treponemal loads and serological results or HIV status. This study suggests a scenario where syphilis may be characterized by: i) heterogeneous and high treponemal loads in primary syphilis, regardless of the anatomic site, reflecting dissimilar duration of chancres development and resolution; ii) high dispersion of bacterial concentrations in secondary syphilis, potentially suggesting replication capability of *T. pallidum* while in the bloodstream; and iii) bacterial evasiveness, either to the host immune system or antibiotic treatment, while remaining hidden in privileged niches. This work highlights the importance of using molecular approaches to study uncultivable human pathogens, such as *T. pallidum*, in the infection process.

1. Introduction

Treponema pallidum subsp. *pallidum* (*T. pallidum*) is a spirochete responsible for the sexually transmitted disease syphilis. This highly adapted human pathogen adheres to epithelial cells and extracellular matrix components of the skin and mucosa, replicates every 30–33 h and is transmitted either by sexual contact, by the mother to the fetus during pregnancy, or by blood transfusion [1,2]. In 2008, the World Health Organization estimated 10.6 million global new cases of syphilis among adults [3], while in Portugal, although underreported, cases doubled from 2013 to 2014 [4]. Syphilis is a multistage disease with a wide-range of manifestations that can be classified as an acquired or congenital disease, divided into early syphilis – which can progress into primary, secondary and early latent infections – and late syphilis – which can progress into late latent or tertiary syphilis [2,5]. While latent syphilis stages are not associated with specific symptoms, in primary syphilis the disease manifests primarily as painless ulcers, developing at an average of 3 weeks after exposure, and in secondary syphilis it

Abbreviations: CSF, cerebrospinal fluid; PBMC, peripheral blood mononuclear cells; qPCR, real-time quantitative PCR.

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manifests as ulcers and mucocutaneous lesions, that can resolve spontaneously after 3–12 weeks [2,6]. Currently, serological tests, divided into non-treponemal tests (Venereal Disease Research Laboratory and Rapid Plasma Reagin) and treponemal tests (*Treponema pallidum* Particle Agglutination test, *Treponema pallidum* haemagglutination test or Enzyme Immunoassays), remain the diagnostic standard for syphilis and performing at least one of each test is sufficient to confirm syphilis [5,7]. Moreover, PCR based techniques are also considered useful tools for diagnosis, particularly using swabs from lesions for the early diagnosis of primary syphilis when a serological response is absent [8–10].

Recently it was possible to access the *in vivo* genomic variability of *T. pallidum* by performing whole-genome sequencing directly from clinical samples [11,12]. Nevertheless, with exception of major antigens, genetic variability was found to be scarce, which anticipates difficulties in the establishment of associations between genomic features and disease outcomes. Given that the factors leading to the different manifestations of the disease are still unknown, obtaining quantitative information regarding the treponemal load throughout the infection may help understand the differential disease outcomes and progression. In this context, we aimed to assess the association between *T. pallidum* loads in distinct clinical samples and dissimilar syphilis disease outcomes through a retrospective cross-sectional study, in order to shed light on *T. pallidum* infections and ultimately, to better understand syphilis.

2. Materials and methods

2.1. Biological specimens

This is a retrospective cross-sectional study enrolling samples available at our laboratory at the Portuguese National Institute of Health that had been previously subjected to syphilis diagnosis. All samples had tested positive for syphilis by using either a nested-PCR, as previously described [13] or the commercial kit *Treponema pallidum* Real-TM (Sacace Biotechnologies, Como, Italy). The vast majority (94%) of these samples had been collected from individuals attending the major Portuguese sexually transmitted disease clinic (Lapa Health Centre, Lisbon, Portugal) from 2004 through 2015, and all individuals presented clinical signs of syphilis (Table 1). Samples had been collected from diverse anatomic sites, namely penis, anus, vagina, scrotum, lip, oropharynx and tongue lesions (all swab samples), cerebrospinal fluid (CSF), vitreous humor, placenta and whole blood or isolated peripheral blood mononuclear cells (PBMC). Results for serological tests (non-treponemic VDRL test and treponemic TPHA test) that were associated with the enrolled samples were provided by the clinicians. According to the guidelines established in this major STD clinic, at the first consultation all patients presenting syphilis clinical signs were immediately subjected to treatment, i.e., administration of Benzathine penicillin G 2.4 million units intramuscularly.

For the present retrospective cross-sectional study, we used a collection of 309 DNA samples (one sample/patient) from the aforementioned specimens that had yielded positive diagnostic for syphilis. These had been rigorously maintained at −80 °C since diagnosis, assuring minimal degradation. These positive DNA samples were herein subjected to an in-house real-time quantitative assay (see below) in order to assess the association between *T. pallidum* loads and dissimilar syphilis disease outcomes.

No ethical issues are raised as records were anonymized (linked by a specific identification number) to ensure patient confidentiality. Furthermore, an informed consent had been obtained from each patient allowing sampling processing and testing for the necessary assays demanded by the clinicians who authored the present study.

Table 1
Characterization of the studied samples.

Number samples (n = 309)	
Specimen type	
Penile exudates	132
Anal exudates	40
Vaginal exudates	12
Tongue exudates	5
Oropharyngeal exudates	2
Scrotum exudates	2
Lip exudates	1
Blood	59
Isolated peripheral blood mononuclear cells (PBMC)	46
Cerebrospinal fluid (CSF)	7
Placenta	2
Vitreous Humor	1
Syphilis stage	
Primary	200
Secondary	65
Secondary presenting rashes	52
Secondary presenting condylomata lata	13
Early latent	9
Treated[a]	13
Undetermined latent	12
Congenital	3
Neurosyphilis[b]	7
Serology Test (VDRL and TPHA) from patients with:	
Both tests Positive	216
Only VDRL Positive	5
Only TPHA Positive	32
Both tests Negative	37
n.a.	19
Co-Infections with sexually transmitted pathogens[c]	
From patients TP^+/HIV^+/Other STI+/-	*70*
From patients TP^+/HIV^-/Other STI^+	38
From patients TP^+/HIV^-/Other STI^-	201

n.a. – not available.
[a] Considered treated when, after antibiotic administration, clinical sings disappeared and at least a four-fold decrease in the serology titers was observed.
[b] Diagnosis performed by detection of *T. pallidum* DNA in CSF samples.
[c] Namely *Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Mycoplasma genitalium*, Human Immunodeficiency virus (HIV), Human Papilloma virus, Herpes Simplex 1 and 2 virus, Hepatitis B and C virus.

2.2. Generation of standard curves for real-time quantitative PCR (qPCR)

To quantify the number of *T. pallidum* genomes in each sample, a plasmid standard curve was generated as previously described for other pathogens [14,15]. Primers for the highly conserved *T. pallidum* single-copy gene *tprB* were designed based on constant regions (primers TP0011-A GGGAGCAGGATTCAAGATCGT and TP0011-B TTGTACGGTGTCCCAGCATC designed using Primer Express software; Applied Biosystems), according to the sequence of *T. pallidum* Nichols strain (CP004010.2). Briefly, an amplified fragment (51 bp) of *tprB* was cloned into the pCR® 2.1 vector using the TOPO TA technology (Invitrogen, MA, USA) according to the manufacturer's instructions. After DH5α *E. coli* overnight propagation, plasmid DNA was purified and transformation was confirmed by PCR and sequencing. The plasmid copy number was calculated according to the formula: Nº plasmid/μL = (Avogadro's Nº × Plasmid conc. (g/μL))/MW of 1 mol of plasmids (g). The standard curve consisted of eight-serial plasmid dilutions (≈1 to 1×10^8 plasmid copies/μL). The number of human cells/sample was quantified by a similarly generated plasmid standard curve using an amplified fragment (73 bp) of a single copy human gene (*β-actin*) according to Gomes et al. [14] (primers B-actin-3 GGTGCATCTCTGCCTTACAGATC and B-actin-4 ACAGCCTGGATAGCAACGTACAT).