Molecular diagnostics and epidemiology of Treponema pallidum

Zondag, H.C.A.

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Chapter 2

Detection of *Treponema pallidum* DNA during early syphilis stages in peripheral blood, oropharynx, ano-rectum and urine as a proxy for transmissibility

Silvia A. Nieuwenburg*, Hélène C.A. Zondag*, Sylvia M. Bruisten , Vita W. Jongen, Maarten F. Schim van der Loeff, Alje P. van Dam**, Henry J.C. de Vries**

* These authors contributed equally

** These authors contributed equally

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ABSTRACT

Background

Syphilis diagnosis may be challenging, especially in the asymptomatic and early clinical stages. We evaluated the presence of *Treponema pallidum* DNA (TP-DNA) in various sample types to elucidate transmissibility during various syphilis stages.

Methods

The study was conducted at the Amsterdam Centre for Sexual Health. We included adult men who have sex with men (MSM), who were suspected of having syphilis. The 2020 European guidelines definitions were followed for the diagnosis and staging of syphilis. Using a polymerase chain reaction (PCR) targeting the *polA* gene of *Treponema pallidum* (TP-PCR), we tested the following study samples on TP-DNA: peripheral blood, oropharyngeal swab, ano-rectal swab, and urine.

Results

From November 2018 to December 2019 we included 293 MSM. Seventy clients had primary syphilis, 73 secondary syphilis, 86 early latent syphilis, 14 late latent syphilis, 23 treated syphilis, and 27 had no syphilis. TP-DNA was detected in at least 1 study sample in 35/70 clients with primary syphilis (2/70 peripheral blood, 7/70 oropharynx, 13/70 ano-rectum, and 24/70 urine); in 62/73 clients with secondary syphilis (15/73 peripheral blood, 47/73 oropharynx, 37/73 ano-rectum, and 26/73 urine); and in 29/86 clients with early latent syphilis (5/86 peripheral blood, 21/86 oropharynx, 11/86 ano-rectum, and 6/86 urine). TP-DNA was not detected in clients with late latent syphilis or treated syphilis, nor in clients without syphilis.

Conclusions

TP-DNA was frequently detected in various sample types in the absence of lesions. This is in line with the high transmission rate of syphilis and opens diagnostic opportunities for early presymptomatic syphilis stages.

INTRODUCTION

Syphilis is a multistage sexually transmitted disease (STD) caused by the bacterium *Treponema pallidum* subspecies *pallidum* (TP) (1). Worldwide syphilis rates are on the rise (2). Like in many countries in the Western world, in the Netherlands syphilis is predominantly found in men who have sex with men (MSM), and especially in MSM living with HIV (3, 4).

Early syphilis is defined as the infectious stage within the first year after acquisition. Early syphilis can be subdivided in primary syphilis, characterized by the presence of a chancre, secondary syphilis, which is clinically polymorphous, and early latent syphilis, which is symptomless (5). After the first year, spirochetes can persist in untreated patients and cause late symptoms of syphilis. However, the spirochete burden at this late stage is considered to be very low and patients are regarded as not sexually infectious (6). Sexual syphilis acquisition is believed to occur via exposure to infectious genital lesions, but also via lesions in body orifices involved in sexual contact such as the anus and mouth. These lesions are considered highly infectious, with an efficiency of transmission estimated at approximately 30% per sexual act (7). The high incidence of early syphilis among MSM emphasizes the need to detect infected individuals as early as possible to prevent ongoing transmission.

The diagnosis of syphilis remains challenging. A combination of clinical findings, direct demonstration of TP by dark field microscopy or polymerase chain reaction (PCR) tests, or indirect serological tests form the basis for a syphilis diagnosis (8). However, in primary syphilis, serological tests may be negative due to the window period between transmission and seroconversion. Therefore, PCR tests for the detection of *T. pallidum* have been introduced as routine diagnostics in several medical microbiology laboratories worldwide (9-12), including in our public health laboratory in Amsterdam, the Netherlands (13, 14).

The sensitivity of *T. pallidum* PCR (TP-PCR) tests is high in primary syphilitic chancres and secondary stage condyloma lata, but low when used on samples from stage 2 roseoles and peripheral blood samples (9, 14-18). Positive TP-DNA results have also been seen in anal (19) and pharyngeal swabs in patients with primary ulcers on these locations (20, 21). In addition, TP-DNA has been found in blood and urine samples from patients with syphilis (22-26). A recent study performed in Australia showed that TP-DNA was frequently found in lesional samples, but less often in nonlesional samples (27) .

Although it is known that primary and secondary syphilis are highly infectious, it is not well understood how transmission to other patients occurs (28). So far, the extent of secondary spirochetal spread to distant mucosal locations has not been systematically evaluated in patients. The aim of this study is to evaluate the presence of TP-DNA in peripheral blood and body orifices such as the oropharynx, ano-rectum, and urethra (urine used as a proxy for urethra) in patients with early syphilis. This to improve syphilis diagnostic options and elucidate transmissibility during various syphilis stages.

METHODS

Study population and study design

The Center for Sexual Health of the Amsterdam Public Health Service, the Netherlands, is a low-threshold sexually transmitted infection (STI) clinic performing approximately 50,000 consultations annually. Consultations are at the client's own initiative, anonymous and free of charge. From November 2018 through December 2019 participants were recruited among clients aged 18 years and older visiting the STI clinic.

Clients were eligible for participation if they were MSM and (1) had signs and symptoms suggestive of primary syphilis or secondary syphilis, or (2) were diagnosed with early latent or late latent syphilis. Clients belonging to the first group were invited on their first visit (Supplement Figure 1). Patients belonging to the second group were invited at their follow-up clinic visit.

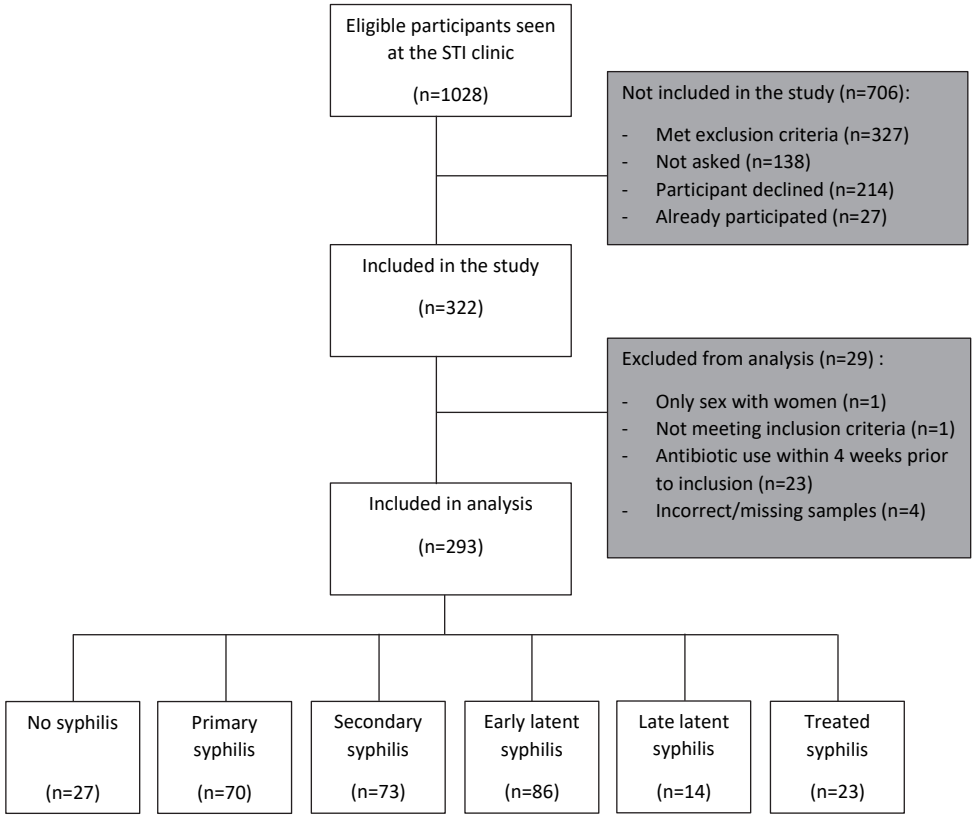

Figure 1 – Flowchart of the inclusion and exclusion of MSM included in the study, Amsterdam, the Netherlands, November 2018 – December 2019.
Abbreviations: MSM, men who have sex with men; STI, sexually transmitted infection.

Upon consent, we collected peripheral blood, client-obtained oropharyngeal and ano-rectal swab, and urine (as proxy for urethral mucosa) for TP-DNA analysis, in addition to the routine diagnostic samples, often including a genital ulcer swab. According to the 2020 European guideline for syphilis (29), participants were allocated into groups based on clinical signs and symptoms, and serological and routine molecular test results using the following algorithm:

1. Primary syphilis: oro- or ano-genital ulcerative disease and a positive dark field microscopy (DFM) or a positive PCR result of the ulcer swab.
2. Secondary syphilis: a rash with or without lymphadenopathy, or mucosal lesions such as condylomata lata, and a rapid plasma reagin (RPR) ≥ 1:4.
3. Early latent syphilis: no symptoms and a seroconversion of the chemoluminence immunoassay (CLIA), or a RPR ≥ 1:32, or a 4-fold or higher RPR titer rise.
4. Late latent syphilis/latent syphilis of unknown duration: no symptoms and no previous syphilis diagnosis and a positive CLIA and a RPR with a titer of <1:32.
5. Findings compatible with treated syphilis (hereafter: treated syphilis): history of previous diagnosis and treatment for syphilis, and not meeting criteria for groups 1 – 4.
6. No syphilis: signs and symptoms compatible with syphilis, but CLIA negative and not meeting criteria for groups 1 – 5.

In the event of a symptomatic participant with a negative initial DFM, negative PCR ulcer swabs and negative serology, but 1 or more positive PCR results in the study samples, such patients were followed up to exclude incubating syphilis. Follow-up would in that case be at 3, 6 and 12 weeks after inclusion with tests at each visit by PCR on peripheral blood, oropharyngeal swab, ano-rectal swab, urine and by serology.

Every client included in this study was examined by a physician for the staging of syphilis. The oral cavity was examined under direct visualization. External examination of the anus was done. If clinically indicated, proctoscopy was done.

Treponema pallidum **diagnostics**

In patients with suspected primary syphilis, DFM on ulcer exudate was performed. Moreover, an ulcer swab for DNA extraction and real-time PCR targeting the *polA* gene was routinely collected (13, 14). This TP-PCR was considered positive when the fluorescent signal was clearly visible and the Cycle threshold (Ct) was <36. For a Ct value between 36 and 40 the DNA extraction was repeated, and both the first and the second DNA extract were (re)tested in the TP-DNA assay. When the Ct value was ≤40, the result was positive for *Treponema pallidum* DNA detection. A Ct value >40 was considered a negative result.

Routine diagnostics at the Public Health Laboratory in Amsterdam include 3 routine serological tests: a treponemal antibody test (CLIA; Diasorin) used for screening and 2 confirmatory tests: the non-treponemal quantitative rapid plasma reagin (RPR) flocculation test (RPR-Nosticon

II; bioMérieux) and the immunoblot (Inno-LIA). The immunoblot was only done if the CLIA value was ≤30. A previous analysis in our department showed that immunoblots were always positive in serum samples with a CLIA value >30 (unpublished data). All serological tests were performed according to the specifications of the manufacturers.

DNA isolation and PCR testing

DNA was extracted from oropharyngeal and ano-rectal dry swab samples using isopropanol precipitation after which the DNA pellet was dissolved in 50 µL of T10 buffer (10 mM Tris-HCl, pH 8.0) (30). From March 2019, the DNA from the swabs was isolated using the MagNA Pure 24 (Roche Molecular Systems), according to the specifications of the manufacturers, after in-house validation to ensure fully comparable results.

DNA from urine and peripheral blood samples was extracted within 24 hours of collection. From the urine samples 1ml was centrifuged at 14000 rpm for 10 minutes. The residual fluid was removed and the pellet resuspended with 200µl PBS and 500 µL easyMAG Lysisbuffer (BioMerieux) containing 0.2% glycogen after which isopropanol precipitation was performed (31). DNA was extracted from peripheral blood using the QIAamp Blood Mini Kit following the protocol from the manufacturer (Qiagen).

All extracted DNA samples were tested using the TP-DNA PCR assay. Simultaneously, all samples were spiked with phocine herpesvirus 1 (PhHV) and tested as inhibition control in the PCR. PCR on DNA extracted from anal swabs frequently showed inhibition and was therefore always simultaneously tested in a 1:10 dilution.

Statistical analysis

We described categorical variables using count data and proportions. Continuous variables were described using their median and interquartile range (IQR). The Fisher exact test was used to compare proportions within categorical variables. The Wilcoxon rank-sum test was used to compare the distribution of continuous variables between groups. We estimated odds ratios (OR) and their 95% confidence intervals (CI) using univariable and multivariable logistic regression to assess associations between determinants and TP-DNA detection in at least 1 sample type. We carried out analyses using Stata (v15.1, StataCorp, College Station, TX, USA).

Ethical statement

This study was approved by the Medical Ethics Committee of the Amsterdam University Medical Center (NL66419.018.18, 2018_236).

RESULTS

Between November 2018 and December 2019, we included 322 participants in the study, of whom 29 participants did not meet the inclusion criteria (Figure 1). Thus 293 MSM were included in the analysis. Among these participants 27 (9%) had no syphilis, 70 (24%) were diagnosed with primary syphilis, 73 (25%) with secondary syphilis, 86 (29%) with early latent syphilis, 14 (5%) with late latent syphilis, and 23 (8%) with treated syphilis. There were no participants with incubating syphilis. Median age was 40 years (IQR 31-48) (Table 1). Of the 293 participants, 167 (57%) were born in the Netherlands. Among the 103 men living with HIV (35%), 93 (90%) used antiretroviral therapy. Of the 190 HIV-negative men, 27 (14%) had used pre-exposure prophylaxis (PrEP) in the preceding year. Thirty-one (11%) of the 293 participants had been notified for syphilis by a sexual partner.

For 35/70 (50%) participants with primary syphilis, TP-DNA was detected in at least 1 study sample: 2 (3%) peripheral blood, 7 (10%) pharyngeal, 13 (19%) anal, and 24 (34%) urine samples (Table 2, Figure 2). For 62/73 (85%) participants with secondary syphilis, TP-DNA was detected in at least 1 study sample: 15 (21%) peripheral blood, 47 (64%) pharyngeal, 37 (51%) anal and 26 (36%) urine samples. For 29/86 (34%) participants with early latent syphilis, TP-DNA was detected in at least 1 study sample: 5 (6%) peripheral blood , 21 (24%) pharyngeal, 11 (13%) anal and 6 (7%) urine samples. No TP-DNA was detected in participants with late latent syphilis or treated syphilis, nor those without syphilis.TP-DNA was detected in 2 or more sample types in 7 (10%) participants with primary syphilis, in 39 (53%) participants with secondary syphilis and in 9 (11%) participants with early latent syphilis (Supplementary Figure 2). Irrespective of syphilis stage, TP-DNA was less frequently detected in peripheral blood samples than in pharyngeal, anal or urine samples (Figure 2). In primary and early latent syphilis the median RPR titer in TP-DNA positive participants was higher than in TP-DNA negative participants, but this difference was significant only in participants with early latent syphilis (p=0.006) (Supplementary Figure 3).

Table 1 – Socio-demographic and clinical characteristics of male patients included in the Trepoli study (n=293) by syphilis stage, Amsterdam, the Netherlands, November 2018 – December 2019

	Syphilis stage											
	No syphilis (n=27)		**Primary (n=70)**		**Secondary (n=73)**		**Early latent (n=86)**		**Late latent (n=14)**		**Treated syphilis (n=23)**	
	n[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]	*n*[1]	*%*[1]
Demographics												
Age (yrs)												
Median [IQR]	35	[27-43]	41	[33-50]	39	[33-48]	40	[30-52]	35	[26-42]	41	[35-48]
<35 years	13	48%	22	31%	24	33%	30	35%	7	50%	5	22%
35 – 44 years	8	30%	21	30%	25	34%	21	24%	6	43%	9	39%
≥45 years	6	22%	27	39%	24	33%	35	41%	1	7%	9	39%
Country of origin												
The Netherlands	16	59%	46	66%	36	50%	51	59%	5	36%	13	57%
Other	11	41%	24	34%	36	50%	35	41%	9	64%	10	43%
Education												
None/primary/secondary	2	8%	15	23%	16	27%	20	25%	4	3%	3	15%
College/university	24	92%	49	75%	41	68%	60	74%	8	67%	16	80%
Other	0	0%	1	2%	3	5%	1	1%	0	0%	1	5%
Sexual behaviour												
Gender of sex partners												
Men	26	96%	68	97%	73	100%	85	99%	13	93%	22	96%
Men and women	1	4%	2	3%	0	0%	1	1%	1	7%	1	4%
No. of sexual partners (6mo)[2]												
Median [IQR]	4	[3-15]	8	[4-20]	6	[4-15]	10	[5-20]	8	[4-12]	8	[5-15]
<5	14	52%	19	27%	23	32%	16	20%	5	36%	5	22%
5-9	4	15%	18	26%	18	25%	21	26%	2	14%	7	30%

10-14	2	7%	11	16%	11	15%	14	18%	4	29%	3	13%
≥15	7	26%	22	31%	21	29%	29	36%	3	21%	8	35%
Health and biometrics												
HIV status												
Negative	21	78%	52	74%	44	60%	49	57%	13	93%	11	48%
Positive	6	22%	18	26%	29	40%	37	43%	1	7%	12	52%
cART use[3]												
No	1	17%	0	0%	3	11%	2	6%	0	0%	0	0%
Yes	5	83%	18	100%	25	89%	33	94%	1	100%	11	100%
Most recent CD4 count (cells/µl)[3]												
<350	0	0%	0	0%	1	5%	0	0%	0	0%	0	0%
350-499	0	0%	0	0%	2	10%	1	3%	0	0%	0	0%
≥500	4	100%	14	100%	17	85%	29	97%	1	100%	8	100%
PrEP use[4]												
No	20	95%	44	85%	37	84%	42	86%	11	85%	9	82%
In the past 3 months	1	8%	8	15%	7	16%	6	12%	1	8%	2	18%
In the past 4-12 months	0	0%	0	0%	0	0%	1	2%	1	8%	0	0%
Chlamydia diagnosis[5]												
No	23	85%	65	93%	69	95%	86	100%	13	93%	19	83%
Yes	4	15%	6	7%	4	5%	0	0%	1	7%	4	17%
LGV diagnosis[5]												
No	24	89%	69	99%	73	100%	86	100%	14	100%	22	96%
Yes	3	11%	1	1%	0	0%	0	0%	0	0%	1	4%
Gonorrhea diagnosis[5]												
No	22	81%	64	91%	67	92%	84	98%	13	93%	19	83%
Yes	5	19%	6	9%	6	8%	2	2%	1	7%	4	17%

Table 1 – Continued

Herpes simplex diagnosis[5]												
No	21	78%	70	100%	72	99%	84	98%	14	100%	18	78%
Yes	6	22%	0	0%	1	1%	2	2%	0	0%	5	22%
Notified for syphilis												
No	23	85%	58	83%	62	85%	86	100%	14	100%	19	83%
Yes	4	15%	12	17%	11	15%	0	0%	0	0%	4	17%
RPR												
Median [IQR]	NA	NA	2	[0-8]	16	[16-32]	8	[4-32]	6	[0-8]	0	[0-1]
Negative	27	100%	21	30%	0	0%	10	12%	4	29%	17	74%
Low (1:1-1:4)	NA	NA	24	34%	4	5%	13	15%	3	21%	5	22%
Middle (1:8-1:16)	NA	NA	15	21%	38	52%	39	45%	7	50%	1	4%
High (1:32-1:128)	NA	NA	10	14%	31	42%	24	28%	0	0%	0	0%

Abbreviations: cART, combination antiretroviral therapy; HIV, human immunodeficiency virus; IQR, interquartile range; LGV, lymphogranuloma venereum; no., number; PrEP, pre-exposure prophylaxis; yrs, years; RPR, rapid plasma reagin

Data missing for: country of origin (n=1), education level (n=29), number of sexual partners (n=6), ART use (n=4), most recent CD4 count (n=26)

1. Unless otherwise stated
2. In the 6 months before the consultation
3. In patients with HIV
4. In HIV negative men only
5. At current visit

Table 2 – TP-DNA detection in Peripheral blood, throat and anal swabs and urine, by syphilis stage, in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Syphilis stage											
	No syphilis (n=27)		**Primary (n=70)**		**Secondary (n=73)**		**Early latent (n=86)**		**Late syphilis (n=14)**		**Treated syphilis (n=23)**	
	n	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*	*n*	*%*
Anatomical location												
Peripheral blood	0	0%	2	3%	15	21%	5	6%	0	0%	0	0%
Throat	0	0%	7	10%	47	64%	21	24%	0	0%	0	0%
Anus	0	0%	13	19%	37	51%	11	13%	0	0%	0	0%
Urine	0	0%	24	34%	26	36%	6	7%	0	0%	0	0%
Number of locations positive												
0	27	100%	35	50%	11	15%	57	66%	14	100%	23	100%
1	0	0%	28	40%	23	32%	20	23%	0	0%	0	0%
2	0	0%	4	6%	21	29%	6	7%	0	0%	0	0%
3	0	0%	2	3%	12	16%	1	1%	0	0%	0	0%
4	0	0%	1	1%	6	8%	2	2%	0	0%	0	0%
≥1 location positive	0	0%	35	50%	62	85%	29	34%	0	0%	0	0%

Abbreviations: MSM, men who have sex with men; No., number; TP-DNA, *Treponema pallidum* DNA.

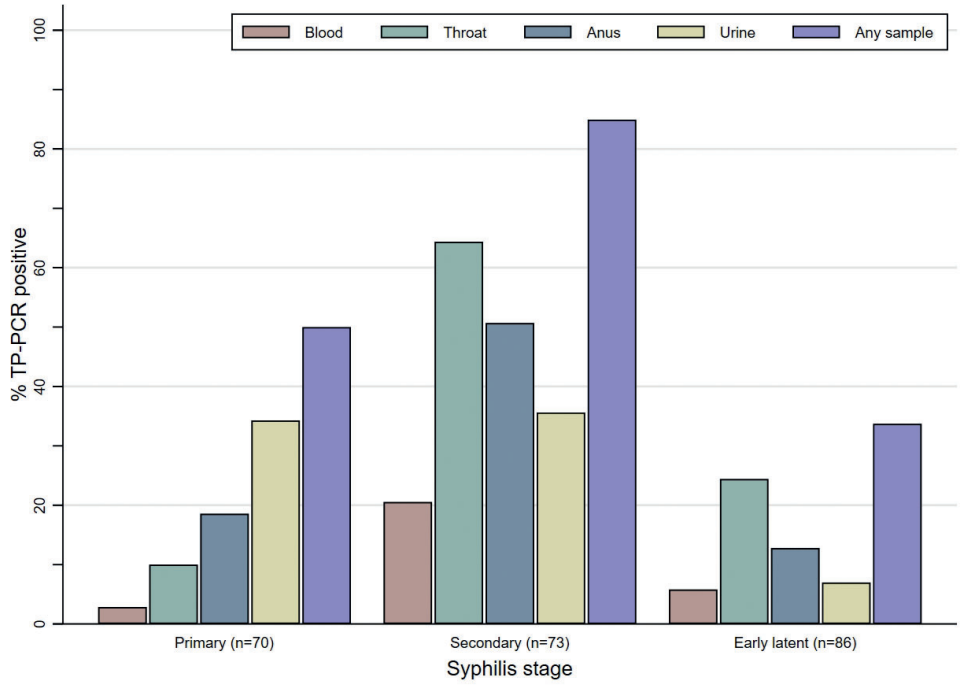

Figure 2 – TP-DNA detection in peripheral blood, throat and anal swabs, and urine in MSM with early syphilis, by stage, Amsterdam, the Netherlands, November 2018 – December 2019.
Abbreviations: MSM, men who have sex with men; TP-DNA, *Treponema pallidum* DNA; TP-PCR, *Treponema pallidum* polymerase chain reaction.

To assess whether TP-DNA positivity in oropharyngeal and ano-rectal swabs and in urine was due to TP-DNA from lesions in patients with ulcerative disease at the site of collection, we separately analyzed those with and without localized ulcerative disease. Among primary syphilis patients without a penile ulcer, 8/29 (28%) urine samples were TP-DNA positive; among secondary syphilis patients without a penile ulcer this was 22/68 (39%) (Table 3, Supplementary Figure 4). Among primary syphilis patients without an anal ulcer, 7/60 (12%) ano-rectal samples were TP-DNA positive; among secondary syphilis patients without an anal ulcer this was 32/68 (47%). Among primary syphilis patients without a pharyngeal ulcer, 6/70 (9%) pharyngeal samples were TP-DNA positive; among secondary syphilis patients without a pharyngeal ulcer this was 46/71 (65%).

Table 3 – Urine, anal and pharyngeal TP-PCR positivity in the presence or absence of ulcers in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Penile ulcer present		**Penile ulcer absent**	
		TP-DNA positive		**TP-DNA positive**
	N	*n (%)*	*N*	*n (%)*
Urine				
Primary syphilis	41	16 (39%)	29	8 (28%)
Secondary syphilis	5	4 (80%)	68	22 (33%)
Early latent syphilis	0	0 (0%)	86	6 (7%)
	Anal ulcer present		**Anal ulcer absent**	
		TP-DNA positive		**TP-DNA positive**
	N	*n (%)*	*N*	*n (%)*
Anus				
Primary syphilis	10	6 (60%)	60	7 (12%)
Secondary syphilis	5	5 (100%)	68	32 (47%)
Early latent syphilis	1	0 (0%)	85	11 (13%)
	Pharyngeal ulcer present		**Pharyngeal ulcer absent**	
		TP-DNA positive		**TP-DNA positive**
	N	*n (%)*	*N*	*n (%)*
Pharyngeal				
Primary syphilis	1	1 (100%)	69	6 (9%)
Secondary syphilis	2	1 (50%)	71	46 (65%)
Early latent syphilis	2	1 (50%)	84	20 (24%)

Abbreviations: MSM, men who have sex with men; No., number; TP-DNA, *Treponema pallidum* DNA.

We assessed the determinants of TP-DNA detection in one or more sample types. In the multivariable model secondary syphilis was significantly associated with TP-DNA positivity (adjusted odds ratio [aOR] 6.83 (95% CI 2.94-15.90, Table 4). Also, fewer sex partners and a negative HIV status were significantly associated with TP-DNA positivity. There were no significant associations between the Ct value (as a measure of sample TP-DNA content) of the various sample types and syphilis stage. The TP-DNA content of the peripheral blood samples however was markedly lower (higher Ct values) than those of the other sample types (Supplementary Table 1). We assessed study sample TP-DNA positivity by duration of symptoms (dichotomized into ≤2 weeks or >2 weeks) in the primary and secondary syphilis subgroups. In almost all combinations of stage and sample type TP-PCR positivity was higher in participants with symptoms lasting >2 weeks, reaching statistical significance for anal TP-PCR positivity in participants with primary syphilis (p=0.042) and throat TP-PCR positivity in participants with secondary syphilis (p=0.038, Supplementary Table 2).

Table 4 – Univariable and multivariable analysis of determinants of TP-DNA detection in one or more sample types in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	TP-DNA detected ≥1 sample type	**Univariable model**			**Multivariable model**		
	n/N (%)[1]	*OR*	*95% CI*	*p-value*	*aOR*	*95% CI*	*p-value*
Syphilis stage							
Primary	35/70 (50%)	REF		<0.001	REF		<0.001
Secondary	62/73 (85%)	5.64	2.55-12.47		6.83	2.94-15.90	
Early latent	29/86 (34%)	0.51	0.27-0.97		0.55	0.27-1.12	
Age, median [IQR][2]	38 [31-48]	0.81	0.03-1.36	0.084	0.86	0.65-1.14	0.304
Age							
<35 years	48/76 (63%)	REF		0.140			
35-44 years	37/67 (55%)	0.72	0.37-1.41				
≥45 years	41/86 (48%)	0.53	0.28-1.00				
Country of birth[3]							
The Netherlands	74/133 (56%)	REF		0.770	REF		0.246
Other	51/95 (54%)	0.92	0.54-1.57		0.68	0.36-1.31	
Number of sex partners, median [IQR][4]	6 [4-15]	0.65	0.47-0.89	0.008	0.69	0.48-1.00	0.046
Number of sex partners							
<5	39/58 (67%)	REF		0.105			
5-9	32/57 (56%)	0.62	0.29-1.33				
10-14	19/36 (53%)	0.54	0.23-1.28				
≥15	33/72 (46%)	0.41	0.20-0.85				
HIV status							
Negative	88/145 (61%)	REF		0.024	REF		0.041
Positive	38/84 (45%)	0.54	0.31-0.92		0.50	0.25-0.98	
Any *C. trachomatis* infection							
No	120/220 (55%)	REF		0.468	REF		0.657
Yes	6/9 (67%)	1.67	0.41-6.83		1.46	0.27-7.85	
Any *N. gonorrhoeae* infection							
No	118/215 (55%)	REF		0.869	REF		0.433
Yes	8/14 (57%)	1.09	0.37-3.27		0.59	0.16-2.19	

Abbreviations: aOR, adjusted odds ratio; CI, confidence interval; HIV, human immunodeficiency virus; IQR, interquartile range ; MSM, men who have sex with men; No., number; OR, odds ratio; Ref, reference; TP-DNA, *Treponema pallidum* DNA.

1. Unless otherwise indicated
2. OR per 10 year increase in age
3. 1 missing
4. OR for each (log+1) increase in number of partners

DISCUSSION

In this study we detected TP-DNA in anal and pharyngeal swabs, in urine samples (as a proxy of urethral mucosa) and in peripheral blood in patients with early stages of syphilis (primary, secondary and early latent syphilis). These findings support the following assumptions about the nature of syphilis: (1) The presence of TP-DNA in mucosal tissue and body fluids of patients with early latent syphilis supports the notion that syphilis is transmissible in the absence of signs or symptoms. (2) In contrast, the absence of TP-DNA in patients with late latent syphilis is in line with the assumption that the late stages are considered not infectious (5). (3) The presence of TP-DNA in peripheral blood samples in all early syphilis stages is in line with the assumption that hematogenous dissemination of the infection occurs soon after inoculation (31, 32). (4) In comparison to patients with either primary or early latent syphilis, patients with secondary syphilis significantly more often had at least 1 or more TP-DNA positive study sample, significantly more often a TP-DNA positive oropharyngeal and peripheral blood sample. This finding supports the notion that secondary syphilis is the most contagious stage of syphilis. (5) Finally, the absence of TP-DNA in patients with treated syphilis and those without syphilis confirms the specificity of the test used to detect TP-DNA. (14) In none of the study participants an extra diagnosis of syphilis was found based on TP-DNA positivity of study samples, compared to routine diagnostics.

Several studies have shown that PCR can be used to detect TP-DNA in whole blood at various syphilis stages (15-17, 24, 25). Here, we used peripheral blood and confirmed TP-PCR positivity, although this was infrequently detected. The presence of TP-DNA in pharyngeal and anal samples in the presence of a lesion has been reported before (19-21). In this study, we also detected TP-DNA in the absence of a lesion. Oral and anal shedding could therefore play a role in the transmission of syphilis. We also detected TP-DNA in urine samples as was reported previously (23, 27). In both Dubourg et al. and Towns et al, urine samples were positive for TP-DNA in respectively 4/25 (16%) and 12/198 (6%) samples. Although urine samples are easy to obtain, they are hardly ever used for syphilis diagnosis, as judged by the low number of studies. Whether TP-DNA in urine originates from uro-genital tract lesions, such as penile ulcers, or from renal filtration or asymptomatic mucosal lesions is unknown.

Towns et al. (27) reported the presence of TP-DNA in both lesion and nonlesional samples. In their study, oral rinses, urine and anal swabs were also analyzed, as well as semen from some participants. However, they did not analyze peripheral blood samples. Similar to our study, they frequently detected TP-DNA in the oral (44%) and anal (29%) cavity of patients with secondary syphilis, although we found higher proportions (64% and 51%, respectively). We also more frequently detected TP-DNA in extra-lesional samples of participants with primary syphilis (50% in our study vs 30% by Towns et al.) and early latent syphilis (34% in our study vs 8% by Towns et al.). We could not confirm a relation between RPR titre and extra-lesional

TP-DNA as found by Towns et al. They did not include late latent and treated infections nor persons with a negative syphilis diagnosis. In addition, compared to Towns et al. we diagnosed fewer patients with extra-genital ulcers which may explain their lower positivity rates.

The strength of this study was that we systematically screened peripheral blood, oropharynx and ano-rectum samples, and urine which may play a role in the transmission of *Treponema pallidum*. This study also has several limitations. The detection of TP-DNA does not prove the presence of viable and potentially infectious bacteria. Future studies should investigate the viability of TP-DNA positive samples, especially found in the oral and anal cavity. Presently this has not been possible because TP culture of direct patient samples has proven to be very difficult. Furthermore, we found that fewer sex partners and a negative HIV status were associated with TP-DNA positivity. This counterintuitive finding could possibly be explained assuming that those with fewer sex partners and/or a negative HIV status seek care at an earlier (i.e. more infectious) stage and respond better to partner notification calls. In patients with early syphilis, we might have missed lesions. Proctoscopy was only done if clinically indicated (e.g. proctitis or intra-anal lesion) and during throat examination ulcers might have been overlooked. Furthermore, the number of patients without syphilis was small, so the demonstrated precision of the specificity of the test is limited. In addition, this study was conducted among an MSM population; it would be worthwhile to examine whether women may have a different pattern of TP-DNA shedding. Finally, we do not know if patients with 2 or more positive TP-DNA samples acquired this from the same sexual partner. However, we found intra-patient homogeneity in an investigation of the *Treponema pallidum* molecular variation within patients (33).

Performing TP- PCR on extra-genital samples could be useful as an additional diagnostic tool, especially to identify early incubating infections within the serological window phase. Blood samples are rarely PCR positive and thus may not be useful in routine screening. Urine samples, and anal and pharyngeal swabs are frequently routinely collected for STI screening for molecular diagnosis of *C. trachomatis* and *N. gonorrhoeae* infections. An additional TP-NAAT assay could possibly help to diagnose early syphilis infections in patients with non-reactive serological test results, who are nevertheless suspected for having syphilis (34). The diagnostic value of TP-DNA assays in routine STI screening of asymptomatic persons at increased risk for syphilis should be further evaluated in larger cohorts and other routine clinic settings.

In conclusion, we frequently found TP-DNA in peripheral blood, oropharyngeal and ano-rectal swabs, and urine collected from MSM diagnosed with early syphilis. Detection of TP-DNA in these sample types provide a better understanding of the infectious nature of early syphilis stages.

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Patient consent Patient consent was obtained for every individual participating in this study

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Conflicts of interest The authors declare that this study was funded by the Public Health Service of Amsterdam. A current study on syphilis at the Public Health Service has received research use only *Treponema pallidum* Transcription Mediated Assay kits by the company HOLOGIC.

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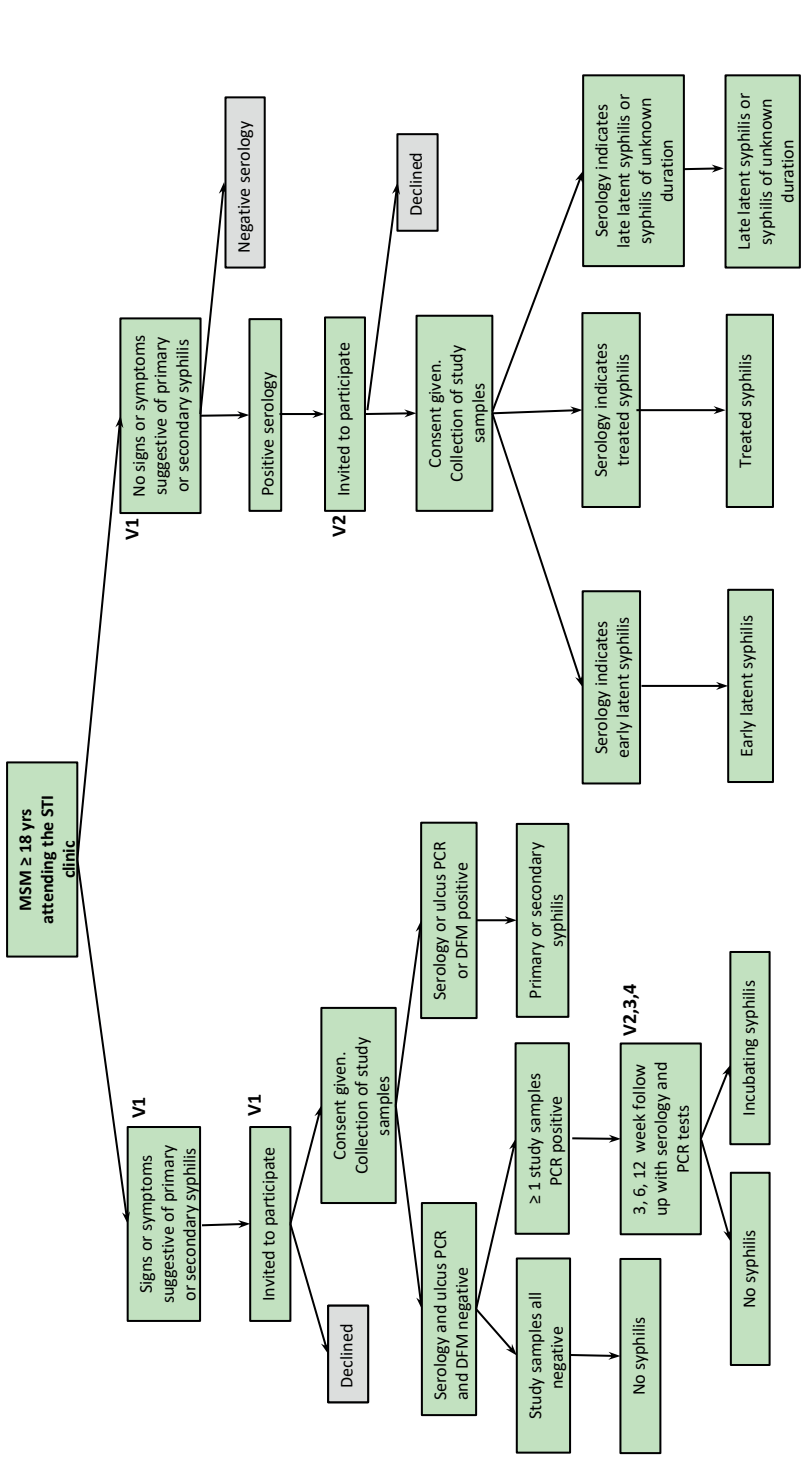
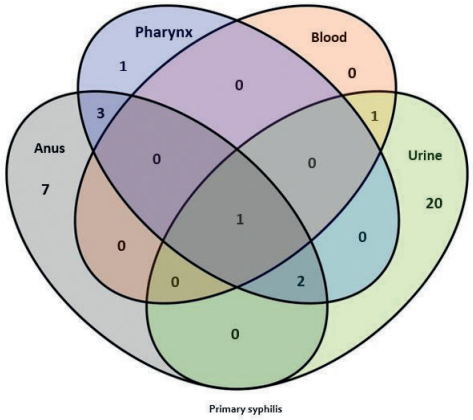
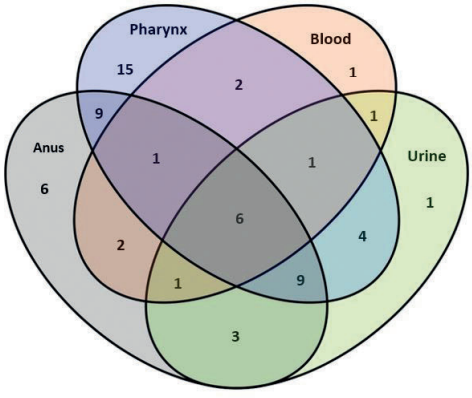
SUPPLEMENTARY DATA



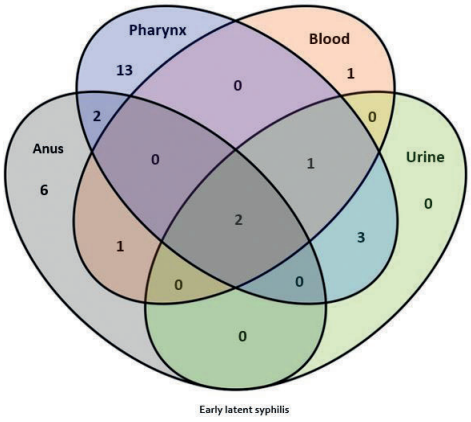
Supplementary Figure 1 – Study design flowchart. Six groups (no syphilis, primary syphilis, secondary syphilis, early latent syphilis, treated syphilis and late latent syphilis or syphilis of unknown duration) were created based on routine diagnostics. DFM: Dark-field microscopy; V: visit.



Primary syphilis

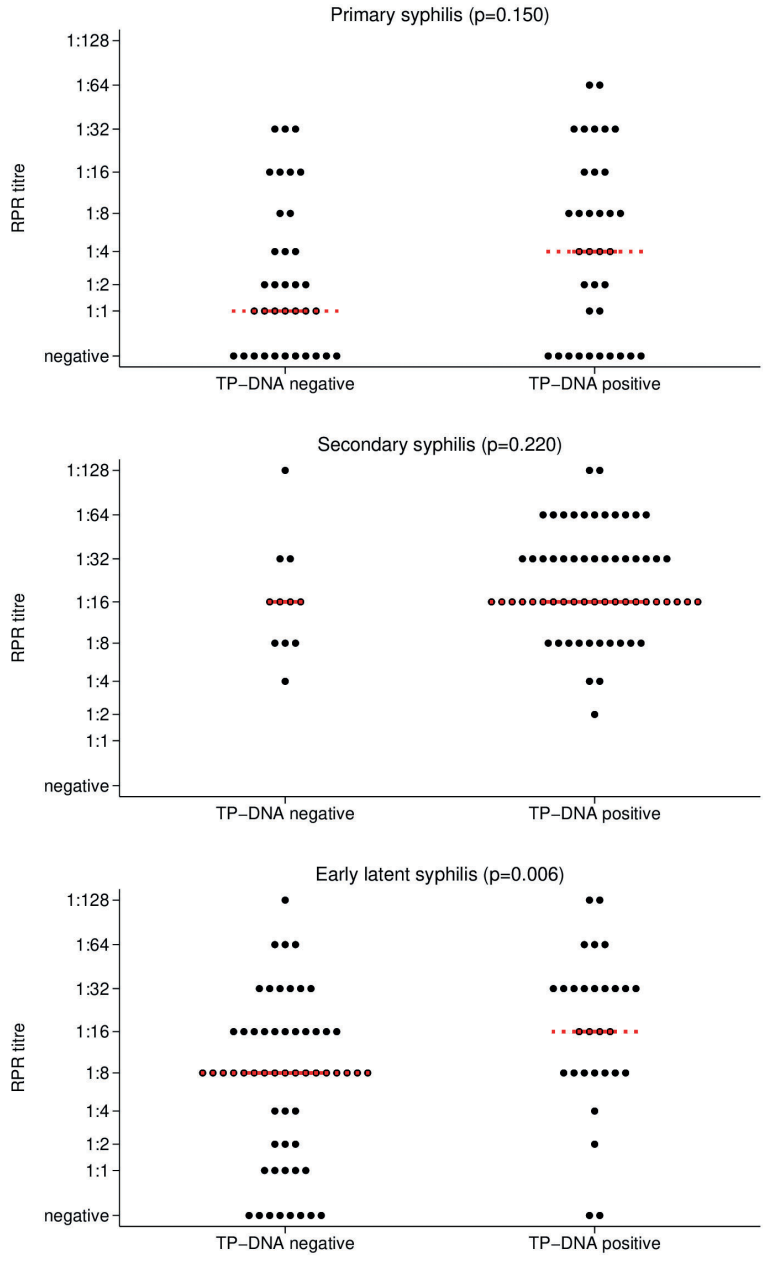


Secondary syphilis

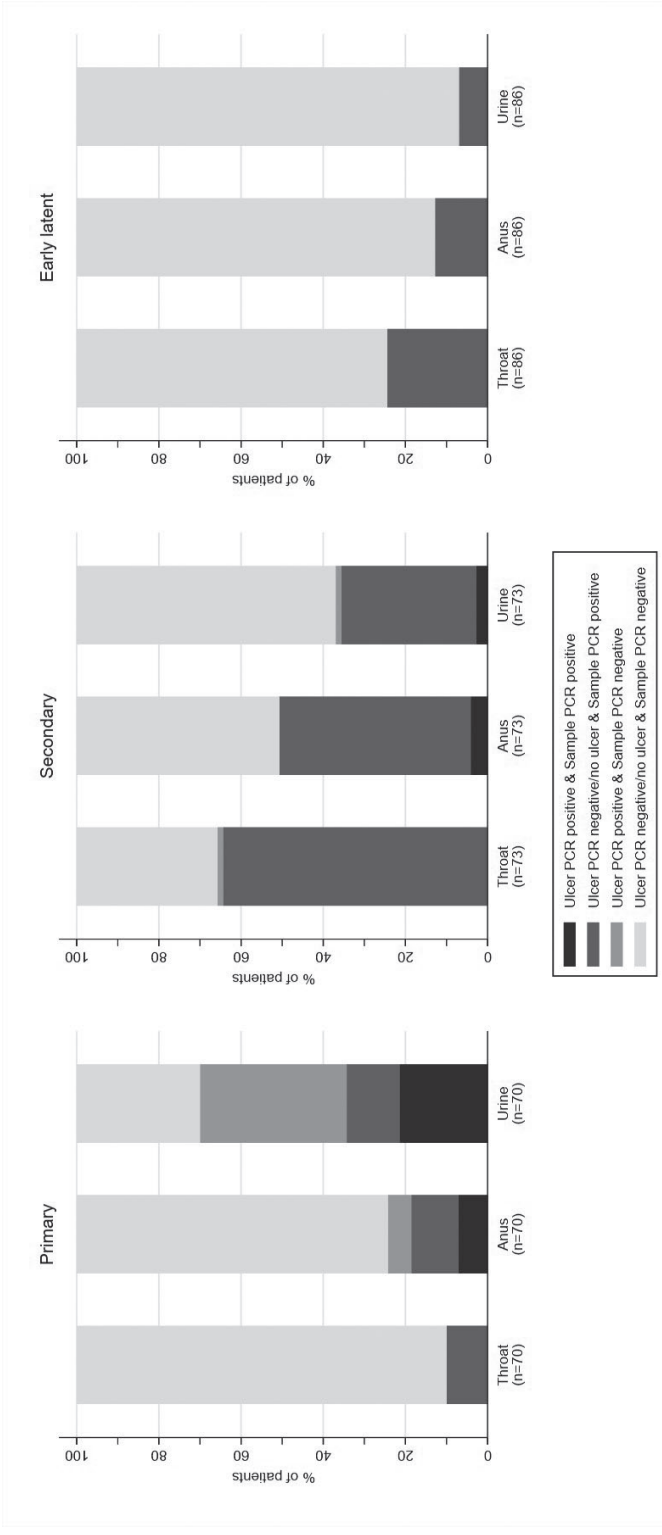


Early latent syphilis

Supplementary Figure 2 – Simultaneous detection of TP-DNA in various sample types ,by syphilis stage, in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019



Supplementary Figure 3 – Non-treponemal antibody titer and the presence of non-lesional TP-DNA in MSM with early syphilis, by syphilis stage, Amsterdam, the Netherlands, November 2018 – December 2019
Red line indicates median



Supplementary Figure 4 – TP-PCR positivity in the presence or absence of ulcers in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

Supplementary Table 1 – Ct values in plasma, throat and anal swabs, and urine, by syphilis stage in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Peripheral blood	**Throat**	**Anus**	**Urine**
	Median [IQR]	*Median [IQR]*	*Median [IQR]*	*Median [IQR]*
Primary	35 [34-36]	36 [27-37]	30 [25-35]	32 [30-36]
Secondary	36 [35-37]	34 [31-36]	33 [29-36]	34 [33-37]
Early latent	36 [35-37]	34 [31-36]	32 [29-36]	32 [32-35]

Abbreviations: IQR, interquartile range

Supplementary Table 2 – Association between duration of symptoms and TP DNA positivity by syphilis stage per sample type in MSM with early syphilis, Amsterdam, the Netherlands, November 2018 – December 2019

	Peripheral blood TP DNA			Throat TP DNA			Anus TP DNA			Urine TP DNA		
	Negative	**Positive**		**Negative**	**Positive**		**Negative**	**Positive**		**Negative**	**Positive**	
	n (%)	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]	*n (%)*	*n (%)*	*p-value*[1]
Primary syphilis (n=51)												
Duration of symptoms			1.000			0.111			0.042			0.694
≤2 weeks	42 (98%)	1 (2%)		41 (95%)	2 (5%)		40 (93%)	3 (7%)		30 (70%)	13 (30%)	
>2 weeks	8 (100%)	0 (0%)		6 (75%)	2 (25%)		5 (63%)	3 (38%)		5 (63%)	3 (38%)	
Secondary syphilis (n=40)												
Duration of symptoms			1.000			0.038			0.731			0.451
≤2 weeks	25 (83%)	5 (17%)		11 (37%)	19 (63%)		17 (57%)	13 (43%)		22 (73%)	8 (27%)	
>2 weeks	8 (80%)	2 (20%)		0 (0%)	10 (100%)		5 (50%)	5 (50%)		6 (60%)	4 (40%)	

p-value estimated using the Fisher's exact test

Data missing for: n= 19 patients with primary syphilis and n=33 patients with secondary syphilis