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Validation of Multiplex Serology for *Treponema pallidum* seroprevalence surveys

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Abstract

Treponema pallidum ssp. *pallidum (T. pallidum)* is a pathogenic bacterium causing syphilis – a sexually transmitted infection that affects millions people per year causing lesions on the skin, mucus membranes, lymph nodes, and in rare cases, can affect the brain, heart and central nervous system, as well as create inflammation all over the body. To aid in investigating spread and severity of syphilis infections, we present the development and validation of *T. pallidum* Multiplex Serology. This methodology is ideally suited to large-scale population-based cohort studies due to its low-cost and high-throughput detection of protein antigens specific to *T. pallidum* – Tp15, Tp17, Tp44 and Tp47. These antigens recombinantly expressed and used with Multiplex Serology to assess serostatus for the pathogen. The assay was validated against sera with known serostatus from previous testing with the commercial assays SERODIA®-TP-PA IgG/IgM by Fijirebio (n = 129) and *recom*Line Treponema IgG, IgM by Mikrogen (n = 195). The sensitivity and specificity of the *T. pallidum* Multiplex Serology assays ranged from 94.7-99.3% (median 97.7%) and 91.3-98.3% (median 97.1%), respectively. High concordance to the reference assays was shown with *kappa* values of 0.87-0.96 (median *kappa* 0.95). Thus, developed assay was shown to be a highly sensitive and specific serological assay designed for application to large prospective population-based cohort studies that will allow investigation into the prevalence of infection and investigate associations with this debilitating disease.

Introduction

T. pallidum is a spirochete bacterium and causative agent of syphilis, a highly contagious and debilitating sexually transmitted infection (STI; Schadinn, F & Hoffmann, 1886). Though it lacks any significant toxins, it is believed to cause tissue damage by activating the host's inflammatory immune response (Radolf, 1996). Syphilis progresses through four stages after infection: primary, secondary, latent, and tertiary (LaFond & Lukehart, 2006). Primary and secondary stages are characterized by a spreading skin rash and/or mucous membrane lesions, typically becoming obvious within the first 8 weeks. Most infections are diagnosed and treated during these stages. The latent stage starts at around 6 months and shows no visible signs or symptoms. From there, the infection may proceed into tertiary stage with significant, irreversible symptoms – i.e. nervous and cardiovascular damage and granulomas in organs.

The small (10 µm by 1.5 µm) bacterium is gram-negative, but lacks an outer membrane lipopolysaccharide (Peeling et al., 2017). The outer membrane contains sparse lipoprotein adhesins for attachment to host membranes (Alderete & Baseman, 1980), and its helical shape allows for a corkscrew motion that penetrates mucous membranes and allows extracellular anchoring. As it lacks lipopolysaccharide, the primary treatment is penicillin. β-lactam antibiotics or macrolides are also effective, but macrolide resistance has recently been identified in a number of geographic regions (World Health Organization, 2016). Vaccines have been largely unsuccessful due to sparse and ambiguously detected outer membrane proteins (< 1% of membrane), while most known novel antigens appear on the inner membrane, thus limiting the response of host antibodies (Tomson et al., 2007). Antigenic variation of outer proteins – e.g. TprK, a putative surface-exposed protein - is likely to further contribute to immune evasion (Giacani et al., 2010). Thus, it is important to understand the epidemiology of syphilis to reduce spread and improve patient outcomes.

Each year, there are an estimated 6 million new cases of syphilis, and has been increasing each year (Korenromp et al., 2018). Untreated syphilis has a high mortality rate (>60%, but rarely outside the tertiary stage in adults), and can be vertically transmitted during pregnancy, with 40-80% infant mortality for those being born to untreated mothers. Low income countries predominately drive both growth and mortality - >90% of new cases and >95% of deaths - due to lack of adequate testing supplies and treatments. Higher income countries show similar rates of increase, but much smaller infected populations. Men show drastically high rates of infection than women, about 5x more cases worldwide. Certain communities – e.g. sex workers and men who have sex with men (MSM) – show a significantly higher infection rate as well. Geographically, rates within these communities drastically differ, such as MSM in New York City showing a 140-fold higher risk than nearby heterosexual men

(Pathela et al., 2011). As such, it is important to understand the epidemiology of syphilis to reduce spread and infection.

Many of the established *T. pallidum* tests are effective on individual patients but lack scalability. Visual confirmation techniques – e.g. darkfield microscopy or agglutination assays (e.g. SERODIA® by Fijirebio) – are simple for individual patients, but epidemiological studies can often recruit thousands of individuals, thus requiring tests with minimal material, time, and cost. Serological tests are more appropriate in this application. Here we introduce a serological assay for syphilis based on Multiplex Serology, a high-throughput, bead-based method for detection of antibodies for multiple specific antigens (Waterboer et al., 2005). Antigens are first expressed as glutathione s-transferase (GST)-fusion proteins in *Escherichia coli* (*E. coli*), then polystyrene beads are loaded with these antigens via a glutathione-casein capture protein on the surface of the beads (Figure 1). The antigens bind immunoglobulins G, M and A (IgG, IgM, and IgA, respectively) in the sample sera, then visualized via a biotinylated secondary antibody and a streptavidin-R-phycoerythrin tertiary antibody. Each bead set emits a distinct fluorescent color when excited, generated from precise ratios of two fluorescent dyes conjugated to the bead. This allows allowing identification of up to 100 different bead sets (hence antigens) per sera sample via flow cytometry. This requires only a small sera sample volume (< 10 µl) and can be done in a single reaction chamber similar to an indirect ELISA. This platform has been widely applied to epidemiological studies over the last 15 years; pathogen-specific antigen panels have been developed for numerous infectious agents or cancer types, including for example human polyomaviruses, hepatitis viruses B and C, and human herpes viruses 1-5 (Brenner et al., 2018, 2019; Gossai et al., 2016).

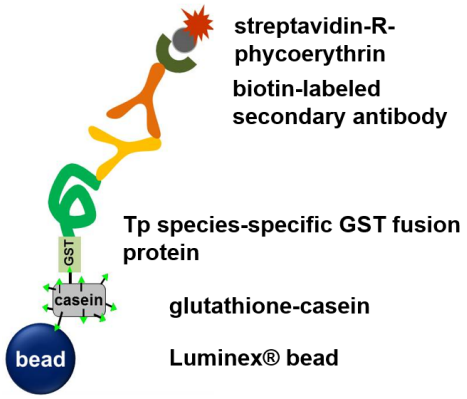


Figure 1. Schematic of multiplex serology bead binding. Treponema species-specific proteins are Tp15, Tp17, Tp44 and Tp47.

Two commercial assays for *T. pallidum* will be used to validate the Multiplex Serology assay: SERODIA®-TP-PA IgG/IgM assay by Fijirebio and *recom*Line Treponema IgG or IgM assay by Mikrogen. SERODIA® uses a particle agglutination assay where a gelatin particle bound with *T. pallidum*-specific antigens is exposed to patient sera. When bound, each particle becomes bulky and disperses across the bottom of a plate well, whereas unbound particles tend to cluster in the middle. This allows an observable difference between positive and negative serostatus (Deguchi et al., 1994).The *recom*Line assay uses immunoblotting via a direct ELISA with bound TP-specific antigen on a paper strip to capture antibodies in patient sera, then detected by anti-IgG/IgM reporter antibodies (Pierro et al.,

2013). Each test uses the presence of numerous *T. pallidum*-specific antigens to determine serostatus. Of the potential intracellular targets to detect seroprevalence, two major studies of the *Treponema* proteome – (Brinkman et al., 2006; McGill et al., 2010) – have identified numerous potential targets with high potential for diagnostic tests: e.g., Tp15 (TP0171) – flavin mononucleotide (FMN) binding; Tp17 (TP0435) – ligand binding, treponemal membrane architecture maintenance; Tp44 (TmpA or TP0768) – unknown function; and Tp47 (TP0547) – carboxypeptidase. Both SERODIA® and *recom*Line use these antigens for detecting presence of *T. pallidum*.

As syphilis is a sexually transmitted infection, it would be useful to compare different infections also transmitted sexually. For use in Multiplex Serology, controls were selected from antigens for human polyoma virus (HpyV; Gossai et al., 2016), herpes simplex virus 2 (HSV-2; Brenner et al., 2018), *Chlamydia trachomatis* (*C. trachomatis;* Trabert et al., 2019), and *Mycoplasma genitalium* (*M. genitalium;* Reichert et al., 2019) were previously expressed. Though not primarily transmitted sexually, there is near universal prevalence (>99%) for at least one strain of HpyVP, so this serves as a strong positive control for useable patient sera (DeCaprio & Garcea, 2013). HSV-2 is an uncurable but effectively managed STI that shows a very low transmission rate, and is often misdiagnosed due to asymptomatic infection and a high rate of false-negatives in commercial tests (Wald & Ashley-Morrow, 2002). Among curable STIs, *C. trachomatis* shows the highest occurrence likely due to high transmission rate and has many highly sensitive, cost efficient tests, thus being a good baseline for STI prevalence among the general population (Malhotra et al., 2013). Similarly, *M. genitalium* is the second most prevalent STI after *C. trachomatis*, is typically asymptomatic, but has no standardized treatment (Gnanadurai & Fifer, 2020).

The aim of this study was to develop a novel Multiplex Serology assay for presence of *T. pallidum* as an indication of past or current syphilis infection using multiple antigens. Specifically, the *Treponema*-specific Tp15, Tp17, Tp44, and Tp47 antigens were used to detect IgG/IgM/IgA levels in human sera. These results were validated against sera previously tested with gold standard reference tests by Fijirebio (SERODIA®) by and Mikrogen (*recom*Line).

Materials and Methods

Materials

Reagents, Kits and Materials: All chemicals were prepared according to the manufacturer's protocol or in double distilled water (ddH_2O), if not indicated otherwise (Table 1-Table **5**). Equipment and software used as specified in manufacturer's instructions (Table 6-Table **7**).

Table 1. Supplies for molecular biology experiments (e.g. PCR, restriction digest)

Name	Supplier
Gel loading dye 6x, Purple	New England Biolabs®
NEBuffer™	New England Biolabs®
peqGREEN (1:10 000)	VWR Peqlab
Restriction endonucleases	New England Biolabs®
SmartLadder 1kbps	Eurogentec

Table 2. Supplies for protein biochemistry experiments (e.g. SDS-Page, Western blot)

Name	Supplier
30% acrylamide mix	Bio-Rad Laboratories
Bradford reagent	Sigma-Aldrich
CBS-K blocking agent	Chemicon International
Clarity Western ECL Substrate	Bio-Rad Laboratories
GelCode™ Blue Stain Reagent	ThermoFischer
MultiScreen® BV	Millipore
PE-Streptavidin Conjugate	MOSS Inc.
Pre-stained Protein Marker	Green BioResearch
Protease inhibitor cocktail	Roche, Mannheim, Germany
TEMED	Bio-Rad Laboratories
Tween-20	Thermofischer

Table 3. Kit systems (e.g. PCR, plasmid isolation)

Name	Supplier
Multiplex PCR Kit	Qiagen; Cat No. 206143
QIAquick Gel Extraction Kit	Qiagen; Cat No. 28704
Q5® High-Fidelity DNA Polymerase	New England Biolabs®; Cat No. M0491
Plasmid Midi Kit	QIAGEN; Cat No. 12145
Plasmid Maxi Kit	QIAGEN; Cat No. 12162

Table 4. Buffers and solutions

Name	Composition
Carbonate Buffer (50 mM)	50 nM Na_2CO_3, 50 mM $NaHCO_3$ (1:4, pH 9.6)
Activated substrate buffer	100 mM NaAc (pH 6), 0.01% (v/v) 3,3',5,5'-Tetramethylbenzidine (TMB), 0.002‰ (v/v) H_2O_2
Ammonium persulphate solution (APS; 10%)	10% (v/v) in ddH_2O
Blocking Buffer (BB)	0.2% (w/v) casein in PBS-T
Dithiothreitol (DTT)	2 mM in ddH_2O
ELISA-Coating Buffer (CB)	2 ng/µl glutathione-casein in 50 mM carbonate buffer
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	0.5 mM in ddH_2O
Transfer Buffer	50 mM Tris-HCl, 360 mM glycine, 0.375% (v/v) SDS, 20% (v/v) methanol in ddH_2O
LB-Ampicillin (LB-Amp)	100 µg/mL ampicillin in LB-media

Phosphate buffered saline (PBS)	2.68 mM KCl, 1.47 mM KH_2PO_4, 137 mM NaCl, 7.98 mM Na_2HPO_4 x $2H_2O$ (pH 7.4)
PBS-Tween (PBS-T)	0.05% (v/v) Tween-20 in PBS (pH 7.4)
Protease inhibitor (20x)	1 tablet in 1 mL ddH2O
PVX buffer	0.5% (v/v) polyvinylalcohol, 0.8% (v/v) polyvinylpyrrolidone in BB
Running Buffer	25 mM Tris, 1.4% (w/v) glycine, 0.1% (w/v) SDS
Stop solution	1M H_2SO_4
Storage Buffer	0.05% sodium azide in BB
Tris-acetate-EDTA (TAE) buffer	40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.5)
Western blot - Blocking Buffer (WB-BB)	10% (w/v) in PBS-T

Table 5. Consumable items

Name	Supplier
MicroWell™ PolySorp® flat bottom plate	Sigma-Aldrich (Cat No. M0661)
96-Well Microplates, Polystyrene, Clear	Greiner Bio-one (Cat No. 650101)
COOH-beads xMAP Technology Microspheres	Luminex

Table 6. Technical equipment

Name	Supplier
Gene Pulser 1652076	Bio-Rad Laboratories
Avestin emulsiflex C5 cell homogenizer	ATA Scientific
Gel Doc™ EZ System	Bio-Rad Laboratories
Multiskan™ GO Microplate Spectrophotometer	Thermo Scientific™
Luminex 200 flow cytometer	Luminex
GeneTouch ™ Thermocycler	Biocompare

Table 7. Software for analysis

Name	Source
CLUSTAL	www.clustal.org
R	www.r-project.org
Excel	www.microsoft.com

Antigens and primers: *T. pallidum* sequences Tp15, Tp17, Tp44, and Tp47 (UniProt, 2019) were codon optimized for expression in *E. coli*, then synthesized and assembled into pGEX4T3tag vectors by Eurofins MWG Operon (Table 8, Figure 2). The plasmids were shipped lyophilized and prepared as per manufacturer instructions. Primers specific to these vectors were previously obtained (Table 9).

Table 8. Antigen specifications for pGEX4T3tag vectors

Antigen	Alternate Name	Uniprot Accession ID	Size (bp)
Tp15	TP0171	P16055	435
Tp17	TP0435	P29722	480
Tp44	TmpA, Tp0768	P07643	1047
Tp47	Tp0547	P29723	1314

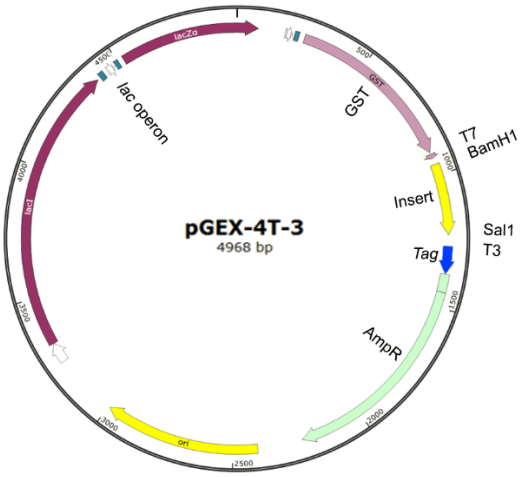


Figure 2. Map of pGEX4T3tag vector. The *T. pallidum* antigen insert is flanked by GST and tag, restriction cut sites for BamHI and Sal1, and T7 and T3 standard primers. A selective marker (ampR) is present, as well as a lac operon for induction of protein expression.

Table 9. pGEX4T3tag primer specifications

Primer	Sequence (5' -> 3')
T3 (reverse)	TAATACGACTCACTATAGGGtccaaaatcggatctggttccgcgtgga
T7 (forward)	AATTAACCCTCACTAAAGGGgatgcggccgcttatgtttcaggttcaggg

Bacterial Strain: The *E. coli* strain BL21 was used for plasmid DNA amplification and isolation, and for protein overexpression and purification.

Antibodies: Antibodies used for immunoblotting, ELISA and Multiplex Serology with the respective dilutions are listed in Table 10.

Table 10. Antibodies specifications

Test	Antibody	Name	Species	Dilution
Western Blot	Primary	GST	rabbit	1:10 000 in WB-BB
	Secondary	anti-rabbit-HRP	goat	1:10 000 in WB-BB
ELISA	Primary	IgG1 anti-tag (KT3)	mouse	1:50 in BB
	Secondary	anti-mouse-HRP	goat	1:10 000 in BB
Multiplex Serology	Secondary	anti-human IgM/IgG/IgA-biotin	goat	1:1 000 in BB
	Secondary	anti-mouse IgM/IgG/IgA-biotin	goat	1:1 000 in BB

Methods

Expression of GST-Antigen-Tag fusion proteins

Antigen selection and vector design: Four sequences from *T. pallidum* were chosen as representative antigens for syphilis infection: Tp15, Tp17, Tp44, and Tp47. These antigens were chosen from previous literature as having the most significant signal-to-noise ratios, known sequences, and low numbers of matching peptides (Brinkman et al., 2006; McGill et al., 2010). The antigen sequences were codon optimized for expression in *E. coli*., synthesized and assembled into pGEX4T3tag vectors by Eurofins MWG Operon (Figure 2). Our construct results in the recombinant expression of a fusion protein of N-terminal Glutathione S-Transferase (GST) and C-terminal truncated SV40 large T-antigen (Tag) assembled as GST-X-Tag in pGEX4T3tag vectors. The GST element increases affinity, stability, and solubility compared to raw antigens (Esposito & Chatterjee, 2006), while the SV40 Tag serves as a control for loading the antigens onto distinct bead sets. The vector also uses ampicillin (ampR) for selection of positive transfection and fusion protein expression is induced by a tac promotor of Isopropyl β- d-1-thiogalactopyranoside (IPTG).

Transformation of *E. coli:* Electrocompetent *E. coli* BL21 cells readily take up constructs coding for the target antigens, thus allowing rapid expression and extraction (Sehr et al., 2001). For transformation, 10 ng diluted plasmid were added to 40 µl ice-thawed *E. coli* cells. The transformation was performed with the BioRad Gene Pulser with the following conditions: U = 2.3 kV; C = 25 µF; R = 200 Ohm. After electrotransformation, 1 ml LB media was added to the *E. coli* cells, and the culture was incubated at 37°C for 1h. For each plasmid, 100 µl and 200 µl culture was plated on LB agar supplemented with 10 µg/mL ampicillin. As a negative control 200 µl LB medium were plated on LB agar. Once dry, the plates were incubated at 37°C overnight.

Protein expression in *E. coli:* For each antigen, three single colonies picked, added to 5 ml LB-Amp, then incubated for approximately 6 h on a shaker at 37°C. A Polymerase Chain Reaction (PCR) quality control was taken for each of the colonies chosen to ensure insert presence (see Quality Control, PCR). For each plasmid, a PCR-positive culture was transferred to 250 ml of LB-Amp, then further incubated as before overnight. Glycerol stocks made from the culture (700 µl sample, 700 µl 50% (v/v) glycerol) and stored at -80°C. A further 50 ml was taken for plasmid purification (see Quality Control, Plasmid Purification). The remaining 200 ml of overnight culture was filled to 1 L with LB-Amp, measured for OD, and diluted to approximately 0.5 OD. Each culture was induced with 500 µl IPTG (0.5 mM) and incubated at RT on a shaker for 6h. Then, the culture was centrifuged at 6000 rotations per minute (RPM) at 4°C for 10 min. Pellets were suspended in 10 mL PBS, then stored at -20°C.

To the thawed pellets from above, 20 µl DTT (final conc. 2mM) and 500 µl protease-inhibitor were added. The suspended cells were then lysed via a cell homogenizer or 'french press' – a hydraulically driven pump that shreds cells by high pressure movement through a needle valve - and centrifuged at 14,000 RPM for 1hr at 4°C until the supernatant lysate was clear. Crude (pre-centrifugation) and clear (post-centrifugation) lysate was stored on ice for later quality control of the proteins (100 µl; see Quality Control, SDS-PAGE). Protein concentration was measured via Bradford protein assay by combining 200 µl Bradford solution, 800 µl H_2O, 1 µl clear lysate; incubate 5 min, then measure OD at 595 nm. Formula for calculation of concentration was obtained from previous studies. Lysates were mixed 1:1 with 100% glycerol, then stored at -20°C. The final lysates were later tested for DNA and protein content (see Quality Control, Sequencing and ELISA).

Quality control

Polymerase Chain Reaction (PCR): Amplification of the inserts by PCR was performed with either QIAGEN Multiplex PCR Kit (Table 11 & Table **12**) or NEB Q5® High-Fidelity PCR Kit (Table 13 &Table **14**) according to the manufacturer's protocol, using the standard primers T3 and T7. As template, DNA toothpick samples of a single colonies were used. Composition of the reaction mix and thermocycler program are shown below. The PCR products were loaded with 10 µl loading dye and visualized on a 1.5% agarose gel (in TAE buffer) with 1:10 000 peqGreen. The DNA fragments were visualized with a Bio-Rad Gel Doc™ EZ System.

Table 11. Multiplex PCR Reaction Mixture

Component	Final concentration	Volume/reaction (µl)
2x QIAGEN Multiplex PCR Master Mix	1x	12.5
5x Q-Solution	0.5x	2.5
Prim_For (10 µM)	0.1 µM	0.25
Prim_Rev (10 µM)	0.1 µM	0.25
ddH2O	-	8.5
Total volume		**25**

Table 12. Multiplex PCR thermocycler program.

Stage	Temp. (°C)	Time (mm:ss)	Cycles
Initial activation	95	15:00	1
Denaturation	94	00:20	
Annealing	68	01:30	44 ↕
Extension	72	01:30	
Final Extension	72	10:00	1

Table 13. Q5® High-Fidelity PCR reaction mix

Component	final concentration	Volume/reaction (µl)
5x Q5 Reaction Buffer	1x	5
5x Q5 High GC Enhancer	1x	5
10 mM dNTPs	0.5 µM	0.5
Forward Primer (10 µM)	0.4 µM	1
Reverse Primer (10 µM)	0.4 µM	1
Q5 DNA Polymerase	0.02 U/µl	0.25
ddH2O	-	12.75
Total volume		25

Table 14. Q5® High-Fidelity PCR thermocycler program.

Stage	Temp. (°C)	Time (mm:ss)	Cycles
Initial activation	99	00:30	1
Denaturation	98	00:15	
Annealing	68	00:30	44 ↕
Extension	72	00:30	
Final Extension	72	10:00	1

Plasmid purification*:* For plasmid purification, the QIAGEN Plasmid Midi and Maxi Kit were used. Due to low availability of QIAGEN-tips, Tr15, Tr17, and Tr47 were purified with the Midi kit, whereas Tr44 was purified with the Maxi kit. The manufacturers protocol was unaltered, and the final elution for all samples was in 50 µl H_2O. Plasmids were then tested via Restriction Digest (see below).

Restriction digest: Samples were diluted in ddH_2O and added to the restriction digest (Table 15). Two digests were performed: a linear digest with BamHI and a symmetric digestion with both BamHI and SalI. The reaction was incubated at 37°C for 1 h, then loaded with 5 µl loading dye and separated on a 2% agarose gel (in TAE buffer) with 1:10000 peqGreen, and visualized.

Table 15. Restriction Digest.

Component	Linear Digest Volume/reaction (µl)	Symmetric Digest Volume/reaction (µl)
10x NEBuffer	2	2
BamHI	1.0	0.5
SalI	-	0.5
DNA	2 (10 ng)	2 (10 ng)
ddH2O	15	15
Total volume	20	20

SDS-polyacrylamide gel electrophoresis (SDS-PAGE): To prepare for further visualization, samples were first prepared by heating to 95°C for 2 minutes to ensure denaturation. An SDS-Page (13.5%

pH 8.8 resolving gel and 4% stacking gel at pH 6.8) was loaded with 10 µl of crude and clear lysate for each *T. pallidum* antigen with 10 µl loading dye along with a sample of raw GST and 5 µl NEB prestained protein ladder (11-245 kDa). The gel ran at 200 V, 400 mA in running buffer for approximately 50 minutes, then blotted for 1 hour at 100 V, 300 mA in transfer buffer. Proteins were then visualized (see Coomassie stain and Western blot)

Table 16. Components of SDS-polyacrylamide gels (2x).

Components	Stacking gel (5%)	Separating gel (13.5%)
ddH_2O	3.675 mL	1.60 mL
1M Tris (pH 6.8)	0.625 mL	-
1M Tris (pH 8.8)	-	3.75 mL
Acrylamide 30	0.665 mL	4.50 mL
10% SDS	50 µl	100 µl
10% APS	25 µl	50 µl
TEMED	5 µl	5 µl

Coomassie stain: For general protein visualization, ThermoFisher GelCode™ Blue Stain Reagent was used to visualize the SDS-PAGE gel: 3 x 5 min ddH_2O wash, 2 h incubation with 20 ml reagent, then a 2 h destain with ddH_2O before visualization.

Western blot: Presence of *T. pallidum*-GST fusion proteins after expression and cell lysis were visualized via Western blot. First, blocking was done with 10% skim milk powder for 1 h, then washed 3 x 5 min with PBS-T. Primary antibody was incubated for 1 h, washed, then the secondary antibody was incubated for 30 minutes. The gel was washed and exposed to Bio-Rad Clarity Western ECL Substrate, then visualized.

Sequencing: To ensure correct inserts in transformed *E. coli*, two samples of each plasmid (25 µl each) were sent for forward and reverse sequencing. A second sequencing run was performed after PCR amplification for 1 ng of template for all plasmids using the Q5 PCR kit as described above. Sequencing was done by Eurofins, and data was analyzed via CLUSTAL.

Enzyme-Linked Immunosorbent Assay (ELISA): To ensure antigen binding capacity, a Polysorp plate was coated with 100 µl/well of glutathione casein 1:1000 in CB and stored overnight at 4°C. The CB was removed, and 180 µl of BB was added to each well, then incubated at 37°C for 1 h. On a Greiner plate, 300 µl dilutions of each plasmid and GST (2 µg/µl each) were plated in the first column of the plate in duplicate. To all other wells, 200 µl of ELISA-BB was added. A serial dilution was performed by taking 100 µl from column 1 and transferring to the next column. This was repeated for columns 1-11 with column 12 remaining as only BB for control. From the Polysorp plate, the blocking buffer was removed, and 100 µl of sample from each well on the Greiner plate was added to the respective well

on the Polysorp plate, then incubated 1 h on a shaker. The plate was washed 3 x 5 min with 0.1% PBS-T, then 100 µl/well of primary antibody was added and incubated 1 h further on a shaker. The plate was washed again as above, then 100 µl/well of secondary antibody in BB and incubated for 1 h on a shaker. After another wash, 100 µl/well of the activated substrate buffer was added. After about 5 minutes, when wells started to turn distinctly blue, 50 µl/well of stop solution (1M) was added. Absorption was measured at 450 nm.

Multiplex Serology

Reference serum selection: The sera used in this validation was obtained from Prof. Dr. Paul Schnitzler at the University Hospital Heidelberg and comprised of sera samples from all syphilis tests performed by the clinic from 2016-2019 (n = 324; median age = 46.7 years (range 2-89 years); 64.1% male). Sera were stored after collection at -20°C. Two different tests were originally used for obtained sera: Fujirebio SERODIA® *Treponema palladium* IgG/IgM particle agglutination assay and Mikrogen *recom*Line Treponema IgG or IgM Assay (Table 17). Routine screening at the clinic was done with SERODIA®, whereas *recom*Line was used for diagnostics; this is due to the significantly higher material cost of immunoblotting with *recom*Line. Due to low test count of *recomLine* IgM, these tests will be combined with *recomLine* IgG. No patient was tested with both reference tests.

Table 17. Reference gold standard test summary statistics. Serostatus is listed as ⊕ for seropositive and ⊖ for seronegative.

Reference test	Assay Type	Immunoglobulins	⊕	⊖
recomLine (Mikrogen)	Immunoblotting	IgG	119	6
		IgM	1	3
SERODIA® (Fujirebio)	Particle Agglutination	IgG/IgM	31	164
		Total	**151**	**173**

Antigen panel and control well selection: Lysates containing known antigens from *T. pallidum* (Tp15, Tp17, Tp44 and Tp47) were used. Additionally, antigens from human Polyoma viruses (VP1 from strains BK, JC, and WU), *C. trachomatis* (pGP-3), HSV-2 (mgG unique) and *M. genitalium* (MgPa N-Term, rMgPa) were used as sera controls.

A blank well - no reference sample - was included on each plate to determine background. Two wells contained only GST to determine the background due to the fusion protein tags. Human standard serum (HuStd; 1:100 and 1:1000 dilution in BB) and polyoma standards serum (PyStd 1:1000 in BB) were also included. KT-3 (mouse SV-40 anti-tag) was as a loading control, diluted 1:25 in BB.

Bead loading: Glutathione casein-coated fluorescence-labelled polystyrene beads were assigned to each antigen with each bead set having a unique fluorescent signature. The bead sets were first thoroughly vortexed to remove clumping. Two dilutions of lysate (1:100 and 1:1000 in dilution buffer) were pipetted into wells containing 2500 beads, then incubated on a shaker in darkness for 1 h at RT.

The beads were washed three times by centrifuging for 2 min at 13 000 RPM, discarding the supernatant, adding 1 ml BB, then resuspending by vortex. The supernatant was again discarded, and 500 µl of storage buffer (LP; BB with 0.05% sodium azide) was added, then stored at 4°C.

Sera Incubation: On a 96 well plate, 2.3 ml diluted GST Tag lysate (2 mg/ml), 2.1 ml of CBS-K blocking agent (Chemicon International Inc.), and 80.6 ml PVX buffer (BB with 0.5% Polyvinylalchol, 0.8% Polyvinylpyrrolidone) was mixed and sera was added to give dilutions of 1:50 and 1:500 in a final volume of 100 µl. The serum dilutions were incubated on a shaker for 1 hr at RT. Next, MultiScreen® wash plates were equilibrated with 100 µl /well H_2O for 10 min at RT, then emptied with a vacuum manifold, and dried using a hammer. The antigen-loaded beads were resuspended by alternating sonification (60 seconds) and vortexing (repeated 6 times). The bead sets were combined (approx. 2500 beads per bead set per well) and added to the wash plates (50 µl each). To each well, 50 µl of serum dilution was transferred, and HuStd, PyStd and KT-3 controls were plated.

Antibody Incubation: The serum pre-incubation was removed with the vacuum manifold, washed (3 x 100 µl BB), and dried with a hammer. The α-human secondary antibody (100 µl) was added to all wells, excluding the anti-tag control. For the anti-tag control, α-mouse was used instead. These were incubated for 1 h at RT. The plates were again washed as stated above. Streptavidin-R-Phycoerythrin (100 µl; 1:750 in BB) was added and incubated for 30 min at RT in the dark. The plate was washed again, then 100 µl BB was added to each well and shaken for 5 min at RT in darkness.

Sera antibody quantification: Each plate was read via a Luminex 200 flow cytometer. Beads within each well are individually analyzed for fluorescence intensity to determine the median fluorescence intensity (MFI) of the beadset in the well. At least 100 beads are tested per bead set per well to ensure accuracy of the measurement.

Analysis

Raw data processing: An R script with a Shiny user interface was developed as an expandable platform for future Multiplex Serology analysis. This was based on a previous developed SAS script and validated against previous studies (data not shown). First, the script combines the patient sera allocation data and beadset identification with the Luminex output into a CSV file, then analyzed via R and Microsoft Excel. From here, wells are excluded due to various conditions: low bead count (< 80), lack of patient sera, excessive GST values, and lack of beadset reactivity. In this study, only 2 samples were removed (both due to lack of patient sera). The cleaned CSV data was loaded back into the R script where GST and plate background are removed from the MFI values, and a correction factor for the Luminex machine used. Further analysis was performed with Excel and R.

Performance Analysis: Classification of serostatus was first evaluated using a receiver operating characteristic (ROC) curve. This uses unblinded serostatus from the reference assays and measures specificity (true positive rate) and 1-sensitivity (false positive rate) of all antigens at all potential MFI cut-off points. Then, measurement of the area-under-the-curve (AUC) indicates the probability of correct binary classification of serostatus. Cut-offs for classification were then derived via optimal Youden's index (J = sensitivity + specificity – 1), then adjusted manually to maximize matches to reference serostatus. Using these cut-offs, performance statistics - sensitivity, specificity, and Cohen's *kappa (k)* - were determined for single antigens and panels of multiple antigens. Cohen's *kappa* measures the agreement between reference and validation assays (e.g. 0.81 < *kappa* < 0.99 means "almost perfect agreement"; Viera & Garrett, 2005).

Sample Sera Analysis: Using the control STI antigens (HSV-2, *M. genitalium*, *C. trachomatis*, and human polyoma virus), the samples were compared to literature values for German populations. The samples were further divided and compared by age and gender.

Results

Quality Control

Colony transformation: The *E. coli* cells were electrotransformed with pGEX4T3tag plasmids containing target antigens and plated on LB agar with ampicillin. The negative control (media only) showed no growth, similar to non-transformed plates (Supplementary Figure 1A & Supplementary **Figure 1**B, respectively). All transformed plates showed growth indicating successful uptake of the plasmids (e.g. Tp44; Supplementary Figure 1C). Colonies were dense, but single colonies were still present.

To verify insert presence, a colony PCR was performed for 3 random isolated colonies from each transformation (Figure 3. **Polymerase Chain Reaction (PCR)**). All samples showed positive for their insert at their expected sizes, with no other bands visible. The bands show a significant difference in intensity between antigens that corresponds with increasing insert size but is consistent between colonies taken from the same plate.

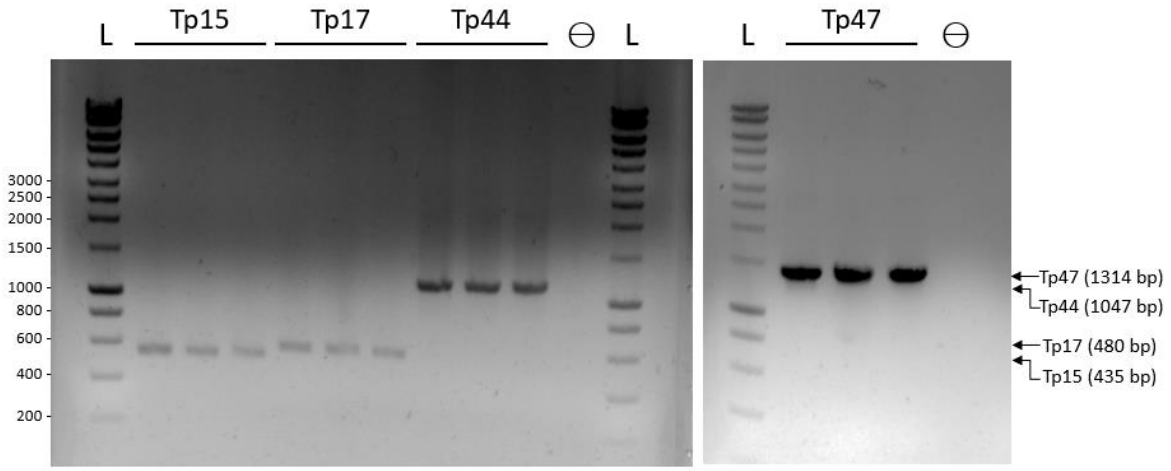


Figure 3. Polymerase Chain Reaction (PCR) for *T.pallidum* inserts (Tp15, Tp17, Tp44, Tp47) after electrotransformation into *E. coli*. All bands shown are the expected size for the respective antigen.

Colony growth: The first colony selected for each plasmid was further incubated into a 250 ml culture until the ODs were within expected range (0.5517 – 0.6471; Table 18). The Tp17 and Tp44 cultures were diluted by replacing 100 ml of the diluted culture with 100 ml of LB-Amp.

Table 18. Absorbance and concentration of culturing, protein expression and sequencing quality controls steps. Tp47 was not sent for post-expression sequencing.

Antigen	Colony Growth		Plasmid Isolation	Protein Expression		Lysate DNA Sequencing	
	OD600	OD600 (diluted)	Concentration (µg/µl)	OD600	Concentration (µg/µl)	Post-expression Conc. (µg/µl)	Post-PCR Conc. (µg/µl)
Tp15	0.5517	-	164.19	0.895	9.9	1930.65	57.97
Tp17	0.6469	0.5319	183.39	0.893	9.9	3492.12	117.46
Tp44	0.6399	0.5359	133.23	1.207	13.2	3360.04	116.09
Tp47	0.6471	-	209.95	1.353	29.8	-	119.08

A Midiprep was taken with 50 ml of sample - all plasmids showed high concentrations (> 133.23 ng/µl; Table 18**Error! Reference source not found.**). A restriction digest was attempted to obtain a linear plasmid (Sal1 or BamHI individually) or asymmetric cuts (Sal1 and BamHI together), but only one faint band was present approximately the expected size of the plasmids (5500-6500 bp), indicating that no double cutting had occurred (Supplementary Figure 2A). This was repeated with 10x DNA (20 µl), but still only one band was present albeit with a much higher band intensity (Supplementary Figure 2B). No further digests were attempted.

Protein visualization: After protein expression, proteins were first examined using a Coomassie stain (Figure 4). Proteins of sufficient content (> 0.1 µg) were dyed, resulting in numerous observable bands. Strong bands were present at all sizes corresponding to the insert + GST size with a slight decrease in band intensity after centrifugation. An observable band for GST was present in all samples, as well as a weaker band at approximately each insert size. Strong secondary bands around 30 and 32 kDa were

present in crude samples of Tp15, Tp17 and Tp44, but faded to background protein intensity after centrifugation.

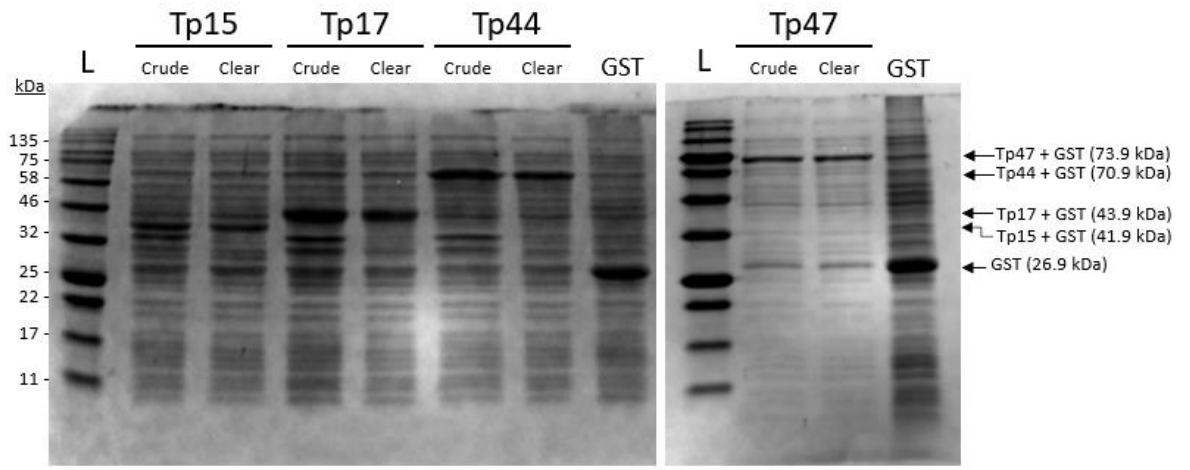


Figure 4. Coomassie stain for *T. pallidum* GST fusion proteins (Tp15, Tp17, Tp44, Tp47) taken from pre- and post-centrifugation (crude and clear, respectively). Strong bands are present at expected sizes for insert + GST. Post-centrifugation shows slight decrease in band intensity for fusion proteins, and drastic drop in unknown proteins around 30-32 kDa in Tp14, Tp17 and Tp44.

Specific protein detection was shown via Western blot using an anti-GST antibody (Figure 5). Strong bands were present at the expected sizes for each fusion protein. Tp47 showed very low protein presence but still easily visible. Like the Coomassie stain, a strong band was present at the expected size of GST for all samples. There was minimal difference in patterns between crude and clear samples in contrast to the Coomassie stain, where numerous bands in the 30-32 kDa band were reduced. Also, a band was present at 58 kDa that is common to all antigens (except Tp47, possibly due to low overall protein content). Some other secondary bands are present in only one fusion protein.

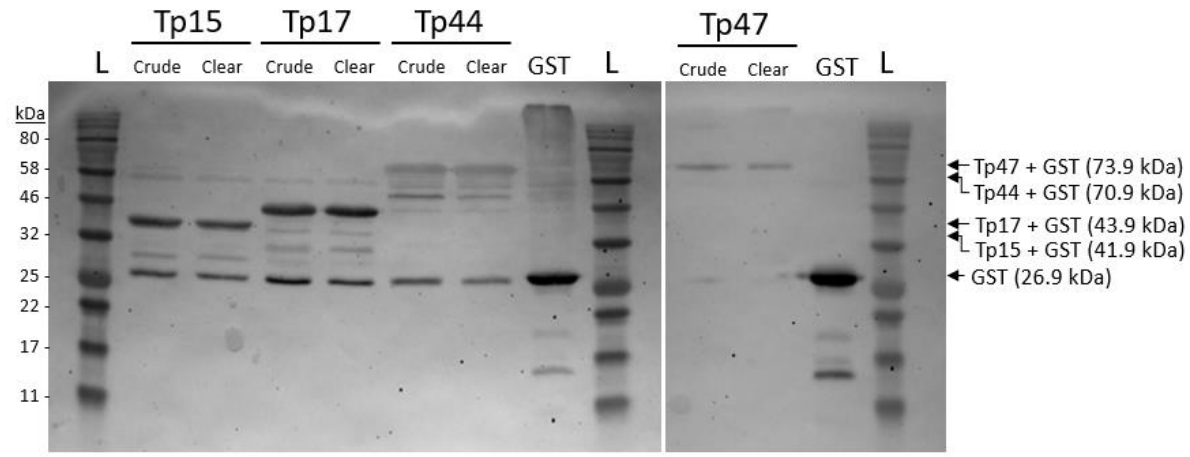


Figure 5. Western blot for *T. pallidum* antigens (Tp15, Tp17, Tp44, Tp47) taken from pre- and post-centrifugation (crude and clear, respectively). Strong bands are present at expected sizes for insert + GST. Post-centrifugation shows slight decrease in band intensity for fusion proteins. A common band is present at 58 kDa, and numerous secondary bands are present at sizes below the fusion proteins.

Sequencing: DNA concentration was measured via Bradford, and all antigen lysates showed high concentration after dilution in glycerol for storage (> 9.9 µg/µl; Table 18). To verify the proteins and ensure successful cloning, sequencing needed to be performed. DNA was isolated via a miniprep, then checked for concentration via Nanodrop before sequencing. The sequencing showed significantly higher quality (average quality > 30 for 85+% of bases; Supplementary Figure 3B) indicating successful cloning.

Antigen binding: An ELISA was performed to check the binding capacity of the GST, and to ensure full length expression of the antigens via the C-terminal tag epitope (Figure 6). All four *T. pallidum* antigens showed results along all dilutions. Maximal binding capacity is unclear for most antigens: Tp15 and Tp17 showed a sharp decrease in absorbance above 50 µg, whereas Tp44 semi-plateaued at about 5 µg, and Tp47 was still unsaturated at 400 µg. Below 1 µg, absorbance levels began to stabilize between the antigens.

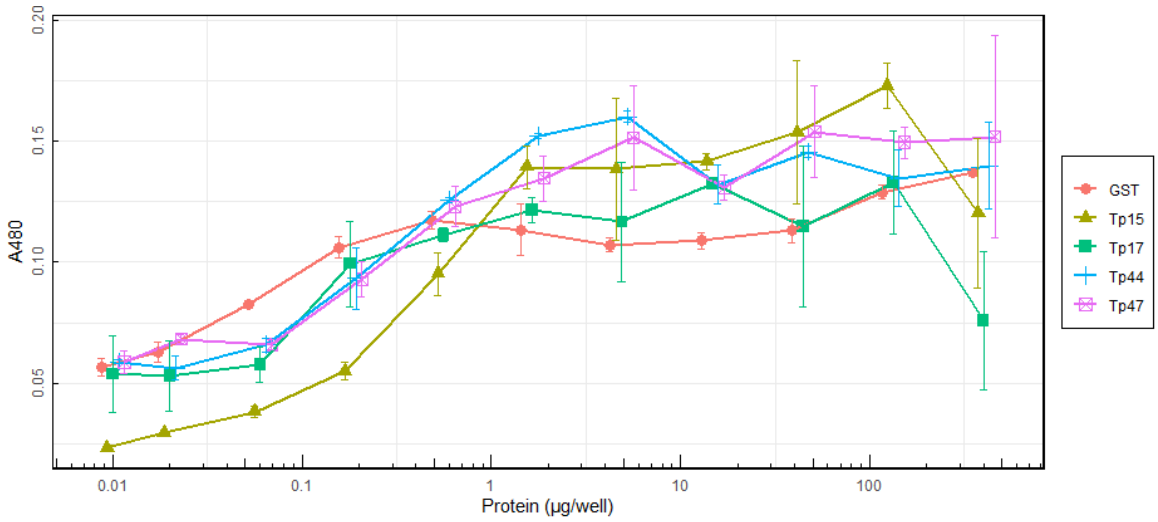


Figure 6. Absorbance of Tp-GST fusion proteins at various protein concentrations. High concentrations of protein showed a decrease in absorbance for Tp15 and Tp17. Tp44 showed a semi-plateau above 5 µg, whereas Tp47 showed no saturation. X-axis values are slightly offset to improve clarity of error bars.

Validation

Luminex reading: The Multiplex Serology was performed as described, and measured via Luminex. Titer levels for *T. pallidum*-specific antibodies for Tp15, Tp17, Tp44 and Tp47 antigens in 324 human sera samples at 1:100 and 1:1000 dilutions were tested here, and compared to previously performed reference gold standard tests *recom*Line *by* Mikrogen (seropositive: 120, seronegative: 9) and SERODIA® by Fujirebio (seropositive: 31, seronegative: 164). All antigens showed successful capture by host antibodies (Figure 7), and bead count was acceptable for all wells (> 80 count for all antigens; Supplementary Figure 4). All *T. pallidum* antigens showed a wide range of intensity that skews toward very low values, as indicated by the undiscernible first quartile of the box plots.

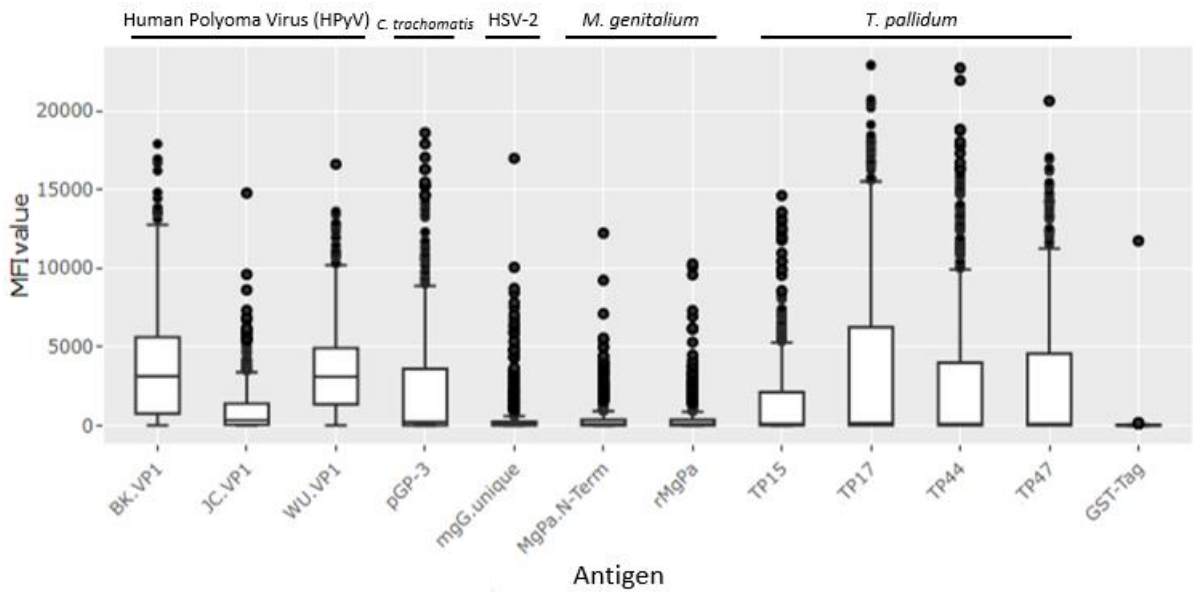


Figure 7. Median fluorescent values (MFI) by Luminex analyzer for IgG/IgM/IgA in sample sera for *T. pallidum* antigens. Most values measured < 5000 MFI, with a heavy skew towards zero. Host pathogen of the respective antigen is indicated at the top. CT = *C. trachomatis*.

Serostatus classification: Serostatus MFI cut-offs were evaluated by ROC (Figure 8A & B). All antigens showed very high AUC values (> 97.1%), suggesting a strong binary classification for positive and negative serostatus. Patients were stratified by reference serostatus, and two distinct populations can be seen for each antigen's respective serostatus (Figure 9). Optimal cut-offs were derived from Youden's index, then manual adjustment to maximize correct serostatus.

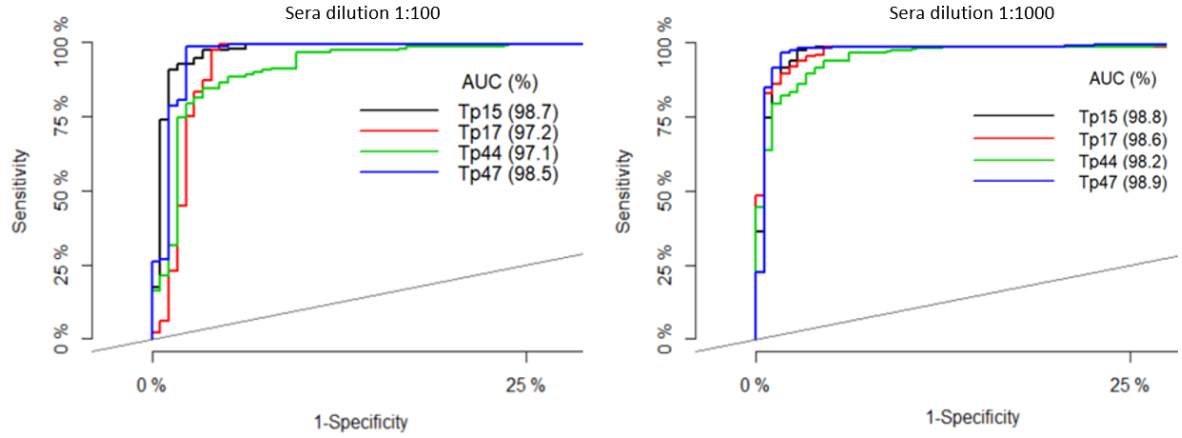


Figure 8. Receiver operating characteristic (ROC) curves for *T. pallidum* antibodies as measured by Multiplex Serology in *T. pallidum* reference sera. Samples were diluted to (A) 1:100 and (B) 1:1000 dilution. Positive/negative reference serostatus was determined by the *T. pallidum* assays SERODIA® (Fujirebio) or *recom*Line (Mikrogen). A random classifier is indicated by the diagonal grey line representing the bisecting line.

Performance characteristics for determined MFI cut-offs using different combinations of antigens were generated (Table 19**Error! Reference source not found.**). All antigens showed high specificity and sensitivity (> 91.3% and > 94.7%, respectively) in comparison to the reference serostatus for both

serum dilutions. Cohen's *kappa* also shows very high agreement with reference serostatus (*kappa* > 0.87). Multiple antigen panel combinations with were tested as well to maximize performance: Tp15 & Tp17 (both dilutions); Tp15, Tp17 and Tp 47; and all 4 antigens. All antigen panels showed improved performance over single antigens (> 0.96), with a panel of Tp15 & Tp17 at 1:1000 showing optimal results – 98.7% sensitivity, 99.4% specificity, and a *kappa* of 0.98. There were minimal false positives and false negatives compared to reference tests (Table 20). Panels of 3 and 4 antigens showed slightly lower sensitivity and specificity, respectively.

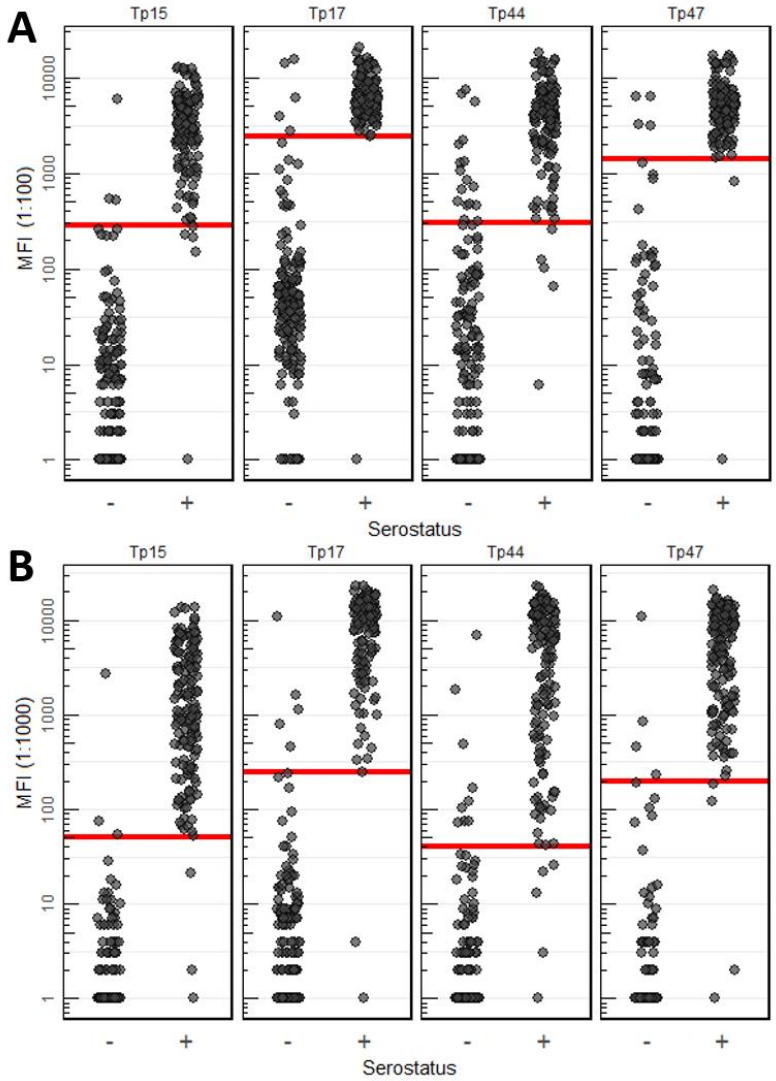


Figure 9. Median fluorescence intensities (MFI) of IgG/IgM/IgA antibodies for *T. pallidum* antigens in patient sera. Sera was diluted to **(A)** 1:100 and **(B)** 1:1000. The T. pallidum antigen against which antibodies were measured in Multiplex Serology is listed at the top of each dot plot. The red line indicates the cut-off for serostatus as determined by Youden's index for the two distinct populations of reference assay seropositive (+) and seronegative (-) serum samples.

Table 19. *T. pallidum* antigen performance in patient sera compared to reference tests. Cutoffs were found by Youden's index (J), then optimized for specificity and sensitivity (Cutoff). 95% confidence intervals (CI) are given. Combination panels are shown with an agreement criterion (≥ N+: seropositive against at least N antigens).

Antigen	Dilution	J (MFI)	Cutoff (MFI)	Criteria	Sensitivity [95% CI]	Specificity [95% CI]	Kappa [95% CI]
Tp15	1:100	279	280	-	96.6 [92.3-98.9]	98.3 [95.0-99.6]	0.95 [0.92-0.98]
	1:1000	53	50	-	98.0 [94.3-99.6]	98.3 [95.1-99.6]	0.96 [0.93-0.99]
Tp17	1:100	2454	2400	-	99.3 [95.5-100]	97.1 [93.4-99.1]	0.96 [0.93-0.99]
	1:1000	251	250	-	98.7 [95.3-99.8]	97.1 [93.4-99.1]	0.96 [0.92-0.99]
Tp44	1:100	279	330	-	96.0 [91.4-98.5]	91.3 [86.1-95.1]	0.87 [0.82-0.92]
	1:1000	42	50	-	94.7 [89.8-97.7]	94.8 [90.4-98.0]	0.89 [0.85-0.94]
Tp47	1:100	1473	1500	-	98.0 [94.2-99.6]	97.7 [94.3-99.4]	0.96 [0.92-0.99]
	1:1000	188	190	-	97.4 [93.4-99.3]	97.1 [93.4-99.1]	0.94 [0.91-0.98]
Tp15 Tp17	1:100	-	-	= 2⊕	99.3 [96.3-100]	96.5 [92.6-98.7]	0.96 [0.92-0.99]
Tp15 Tp17	1:1000	-	-	= 2⊕	98.7 [95.2-99.8]	99.4 [96.8-100]	0.98 [0.96-1.00]
Tp15 Tp17 Tp47	1:1000	-	-	= 3⊕	98.0 [94.2-99.6]	99.4 [96.6-100]	0.97 [0.95-1.00]
All	1:1000	-	-	≥ 3⊕	98.7 [95.2-99.8]	98.8 [95.9-99.9]	0.98 [0.95-1.00]

Sera analysis: Seroprevalence between the patients and previous studies on German populations were compared (Figure 10). The Polyoma control antigens - BK VP1 (90%; Krumbholz et al., 2006), JC VP1 (30-38%; Agostini et al., 2001), WU VP1 (89%; Neske et al., 2010) - showed similar seroprevalence, but the STI antigens for *C. trachomatis* (3.2-5.3% Lallemand et al., 2016), HSV-2 (14%; Pebody et al., 2004), *M. genitalium* (6.5%; Gnanadurai & Fifer, 2020), were drastically increased, especially for those seropositive for *T. pallidum*. Compared to the literature, seropositive patients in this study were 16.5x more likely to be seropositive for *C. trachomatis*. Seronegative patients showed a lower overall STI prevalence compared to seropositive and were close to the public population; *C. trachomatis* was elevated (5.5x), but *M. genitalium* was about even, and HSV-2 was lower (0.5x).

Table 20. 2x2 contingency table for Tp15 and Tp17 (1:1000).

		Reference Assay +	Reference Assay -
Tp15 Tp17	+	147	2
	-	1	172

Cohen's κ = 0.98

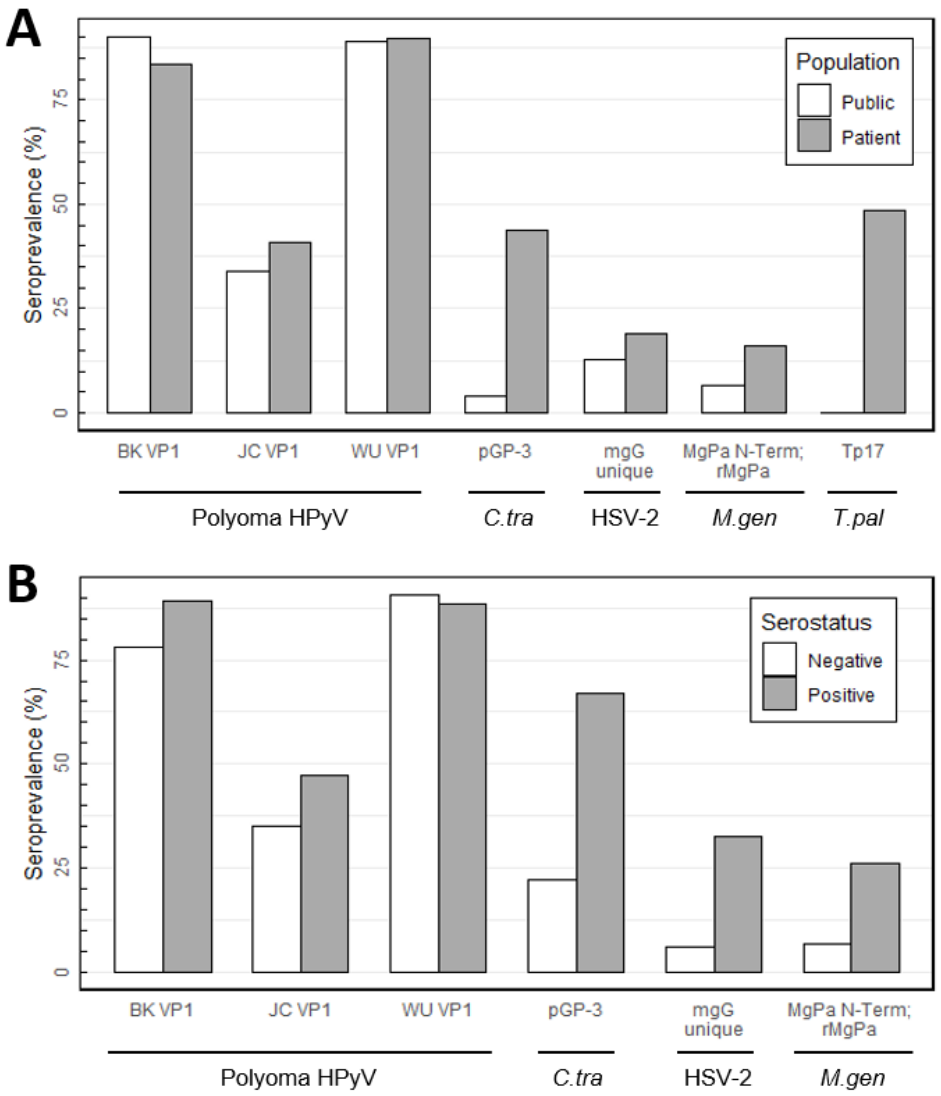


Figure 10. Seroprevalence of control antigens among populations. For control antigens, *C.tra = Chlamydia trachomatis*, *M.gen = Mycoplasma genitalium*, and *T.pal = Treponema pallidum.* **(A)** Patient seroprevalence compared to the general European population (as per previous literature). *T. pallidum* has a general seroprevalence in Germany of ~0.1%. Non-STI antigens (BK VP1, JC VP1, WU VP1) show similar seroprevalence between populations, but patients show highly elevated seroprevalence for STI antigens. **(B)** Seroprevalence for control antigens between seropositive (n = 151) and seronegative (n = 171) sample sera. As before, non-STI antigens show similar seroprevalence between sera, but the seropositive show highly elevated prevalence of STI antigens.

Demographics of the patient population also show obvious differences (Figure 11). Females were less present than men (87 vs 208, respectively), and were far less likely to be seropositive than men (16% vs 55%, respectively). Males showed a significant curve of increasing seroprevalence until a peak age of 51-60 years, and sharply decreasing after. Women showed a near flat seroprevalence curve across all age groups, but a noticeable spike at 51-60 years for seronegativity. Both groups showed similar seroprevalence in both young (0-20 years) and old (80+ years) age groups, as well as a similar mean age (46.0 and 48.2 for males and females, respectively).

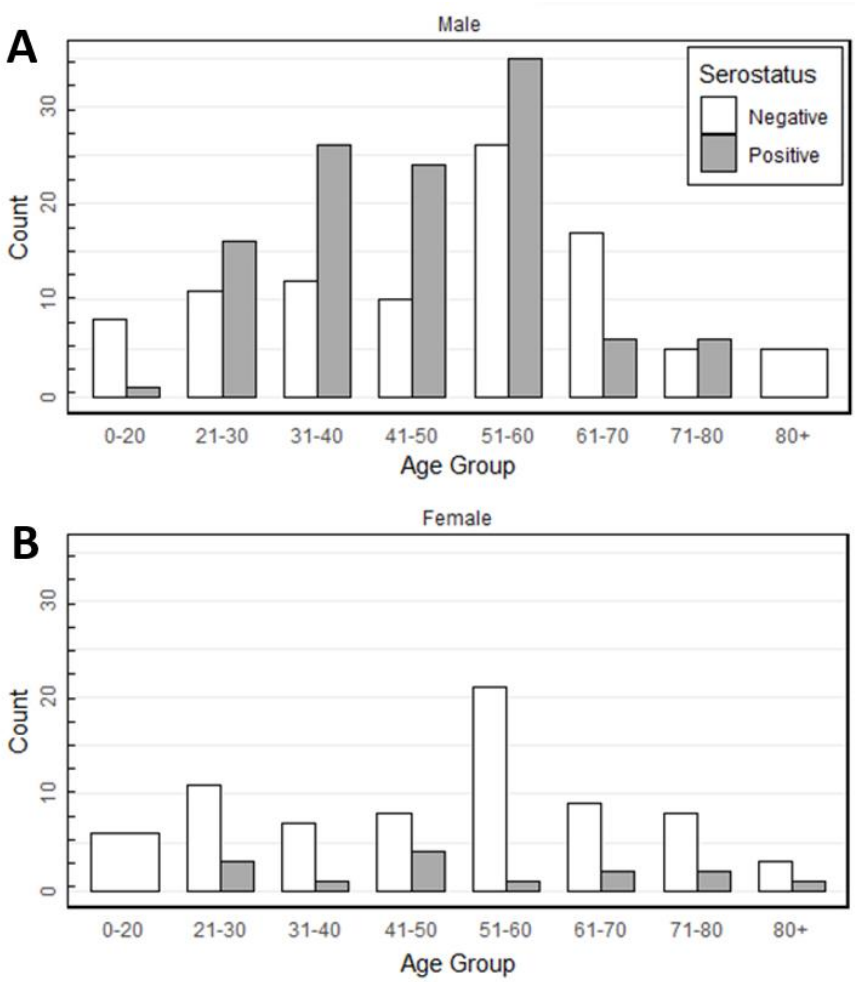


Figure 11. Seroprevalence of *T. pallidum* by age among males (n = 208) and females (n = 87) based on multiplex assay. For **(A)** males and **(B)** females, seropositivity was 55% and 16%, respectively. Females showed a steady low level of seropositivity, while males showed an increasing seropositivity until 60 years. Ends of the age groups (< 20 and > 80 years) showed very low seropositivity.

Discussion

This validation study showed strong performance for a Multiplex Serology assay detecting *T. pallidum* antibodies in agreement with gold standard reference tests from Fijirebio (SERODIA®) and Mikrogen (*recom*Line). All four *T. pallidum* antigens – Tp15, Tp17, Tp44, and Tp47 - can be used effectively alone or in panels at either dilutions (0.87 > *kappa* > 0.98).

During development, the quality control steps were generally successful. Electrotransformation of *E. coli* showed successful uptake of the plasmid as well as a clean PCR of the inserts. The restriction digest failed; the digestion pair - Sal1 and BamHI - are known to work together without inhibition (Suhandynata et al., 2016), and the pGEX4T3tag vector includes both restriction sites, so it is likely some experimental error. The sequencing showed good results (Q score > 30 for > 80% of bases) when using the PCR product of the plasmids. The Coomassie-stained SDS-PAGE gel showed strong protein expression with numerous bands on the gel, and particularly strong bands in our target size for

T. pallidum antigens. The Western blot showed strong expression of our target antigens but numerous unknown bands were present. With *T. pallidum,* different strains can contain alternate proteins; In the Nichols strain, a 13 kDa protein has been observed with > 90% identity with the established 17kDa Tp17 protein (*UPI00051FF3D3*, n.d.). So, an alternate splice site (though unlikely in such a small protein) or post-translational cleavage could cause the truncated protein, in addition to the normal Western blot difficulties that cause multiple bands: i.e. excess lysate, protein degradation, incomplete translation, or proteases being present. For the ELISA, a curve that increased in absorbance as the protein content increased was expected, but two antigens (Tp15 and Tp17) showed a large drop at high content. This might indicate a prozone effect, where excess free antigen blocks the primary antibody from binding to the plate-bound antigen (Tate & Ward, 2004). While this is much more common in a sandwich ELISA, rather than our direct ELISA, inadequate washing could have the same result. Repeating the assay using a lower starting protein content (< 200 µg) may resolve this. Finally, the Multiplex Serology results were positive. All antigens showed a large range of MFI values, and all bead counts were acceptable.

For measuring assay performance, each of the *T. pallidum* antigens showed a near ideal ROC curve for a binary classifier, indicating a high level of antibody response and confirmed by a corresponding high MFI value during the assay. Strongest performance was found to be an antigen panel of Tp15 and Tp17 at a dilution of 1:1000 (specificity and sensitivity of 98.7% and 99.4%, respectively, with a *kappa* of 0.98), but the assay could be reasonably performed at either dilution or with a single antigen without significant loss of performance; even the weakest antigen (Tp44 at 1:100 dilution) showed a sensitivity and specificity of 94.7% and 94.8%, respectively, and a *kappa* of 0.87, still within range of "almost perfect agreement". Hence, this assay is shows flexibility when used along with antigens for different diseases that have better resolution at a certain dilution (e.g. VZV at 1:100; Brenner et al., 2018). This will minimize reagents for the assay and later analysis efforts.

There did not appear to be much difference on which reference test the sera were tested against. The *recom*Line test uses either IgG or IgM, though there are very few IgM tests to analyze (n = 4). IgM levels in sera start rising immediately after infection, peak at 7 days, and near disappear by 35 days, whereas IgG levels start rising after 7 days, peak at 28 days, then plateaus slightly below the peak after 35 days. Theoretically, this could be a disadvantage to the *recom*Line IgM test, as it may only be effective for the first 28 days of infection whereas the secondary (symptomatic) stage of syphilis can last upwards of 6 months. Our assay uses a triple-specific secondary antibody targeting IgG, IgM, and IgA, so it is unlikely to have this problem. The strongest panel in our assay (Tp15 & Tp17 at 1:100) showed only 3 incorrect tests as validated by the reference tests. Two of those are likely outliers (both tested with *recomLine* and discussed later), and the third is very near the serostatus classification

thresholds so the patient may naturally show lower seroreactivity to *T. pallidum*. Though, this serum was tested against SERODIA®, which relies on a visual observation of particle agglutination, so an experienced technician may be able to better distinguish low seroreactivity.

For comparison to existing tests, a similar assay using a bead-based multiple serology protocol has been developed for *T. pallidum* ssp. *pertenue*, the causative agent for yaws (Cooley et al., 2016). Yaws differs from syphilis in that it is not sexually transmitted and typically affects the skin, bone and joints most often in children from rural, warm, tropical areas. This study used only rp17 (analogue to Tp17) and TmpA (Tp44), but should still allow comparison, as ssp. *pertenue* and ssp. *pallidum* are serologically indistinguishable (Perine et al., 1984). The *T. pallidum* antigen construct differed by using an N-terminal beta-galactosidase instead of Tag for testing the binding capacity and ensuring full length expression. Patient samples in this study were stored as either sera from Ghana (n = 255) and Papua New Guinea (n = 163) or dried blood drops from Vanuatu (n = 169). Storage conditions for the sera were not specified. For validation, it was tested against two assays: SERODIA® particle agglutination assay (Fijirebio), as used in our study, and the WAMPOLE® rapid plasmin regain (Alere). Sensitivity and specificity compared to the were slightly lower overall with rp17 than was observed here (90.1% and 97.6%, respectively). Tp44 showed poor sensitivity but high specificity (67.47% and 99.2%, respectively). Validation compared to the RPR test showed similar results. Samples tested from dried blood drops showed much lower performance. The patient population used for testing is highly biased (children aged 5-14 in rural Africa), but is sufficient for validation.

Bias in sera samples is also present in this project. Due to the source of the patient antigens – suspected syphilis infections treated by University Hospital Heidelberg from 2016-19 – it was not our aim to compare seroprevalence to the general public, but can be done for the purposes of this report. The similar seroprevalence between the public and sample populations for the polyoma control antigens suggest similarity between the two groups. It is not typically transmitted sexually, but other mechanisms are not firmly established. This suggests the two populations have similar backgrounds, and is likely due to both populations being located in Germany, but this data was not collected during sera collection. The remaining pathogens are transmitted primarily by sexual contact, and seroprevalence was highly elevated in the sample population. As the inclusion criteria for the sample sera was being tested for an STI (syphilis via *T. pallidum*), it follows that general STIs are is increased in this population. STI rates are substantially reduced by regular condom usage (Holmes et al., 2004), so this could suggest significantly higher rate of unprotected sexual activity within the seropositive patients, and less so in the seronegative patients, compared to the public. This, however, does not reflect the overall level of sexual activity, as sexually active people show a higher rate of condom usage

(Yamamoto et al., 2018). As well, lesions on exposed genital mucosa/skin can transmit STI vectors regardless of condom usage (e.g. oral-oral transmission for HSV).

Demographics for the patient population gives possible insight into the seroprevalence of the tested STIs. Males were more likely to be tested, as well as having a much higher rate of seropositivity. This is consistent with observed incidence of syphilis in Germany as men show a 16x higher rate of infection versus women (Bremer et al., 2017). Men who have sex with men (MSM) are at particularly high risk, showing 85% of new cases. Anal intercourse has long been known to increase transmission rates of STIs due to vulnerable tissues in the anus and rectum, as well as the lack of natural lubrication, but this can largely be reduced by condom usage (Werner et al., 2018). Sexual behaviors regarding condom usage are likely a large factor in transmission, especially with HIV-positive men who self-report an ~80% rate of condomless anal intercourse with non-steady partners, compared to ~30% of untested or HIV-negative men (Jansen et al., 2016). Another factor for high detection in MSM is the increased rate of STI testing due to risk-adapted screening measures, as recommended by the Association of the Scientific Medical Societies in Germany (Bremer et al., 2015). Age also plays a factor in condom usage - rates decrease as age increases – likely due to increased education in safe sex practices in young people and accessibility of protection (Copen, 2017). This is consistent with seroprevalence in the patient population, with older men (peak at 41-50 years) having a much higher risk. No data was obtained about patient sexual practices, but gender and age seroprevalence suggests high correlation with previous literature.

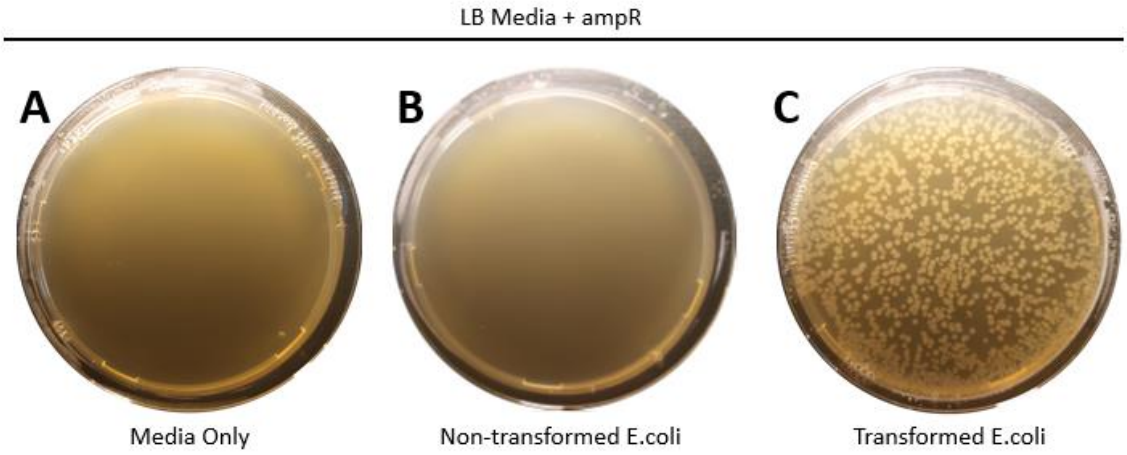
There are some criticisms to this study. First, there is high bias in the study population - patients all had a suspected syphilis infection that necessitated testing. It follows that these individuals had noticeable symptoms before seeking treatment. It has been observed that syphilis can occur asymptomatically (especially in HIV-positive patients; Cohen et al., 2005), so it is unknown whether this assay would be effective to detect these infections. It is possible that *T. pallidum* antigens are expressed differentially given an immunodeficient environment. Commercial tests (like those used here as gold standard) are used during routine HIV-panels, so this assay would likely be effective as well, but would be recommended to use multiple *T. pallidum* antigens to maximize performance in the case of differential expression in immune-compromised patients. Second, antibody response is not consistent. The response is maximized during the first 6-12 months of infection, decreases to a baseline within 2-5 years, and may spontaneously serorevert afterwards (Miller et al., 1999), so this assay may not be effective after the secondary stage. Though, due to obvious physical lesions, these infections were likely diagnosed corresponding to the appearance of first symptoms at up to 3 months (Kent & Romanelli, 2008). Reactivity rates could also play a factor – some patients show inherently low reactivity. The chosen antigens show a high average signal-to-noise ratio, but can also show a high

standard deviation (e.g. Tp17 in sera had a 9.65 signal-to-noise ratio with 4.85 standard deviation; Brinkman et al., 2006). Third, there is evidence of extreme outliers for 3 of the false results: two shows MFI values between 10x and 50x greater than the observed binary classifying cutoff but were tested as seronegative by the gold standard test, while the remaining sample shows MFI values of less than 10 for each *T. pallidum* antigen but was marked seropositive by the reference test. Each of these sera samples was tested with a different test (SERODIA®, *recom*Line IgM, and *recom*Line IgG, respectively), so is likely to be experimental error rather than deficiencies in the test. These patient sera could be re-tested using a different reference assay to confirm serostatus. Despite these criticisms, the assay showed very strong performance for detecting seropositive patients for *T. pallidum* and should prove to be a valuable tool in future epidemiological studies.

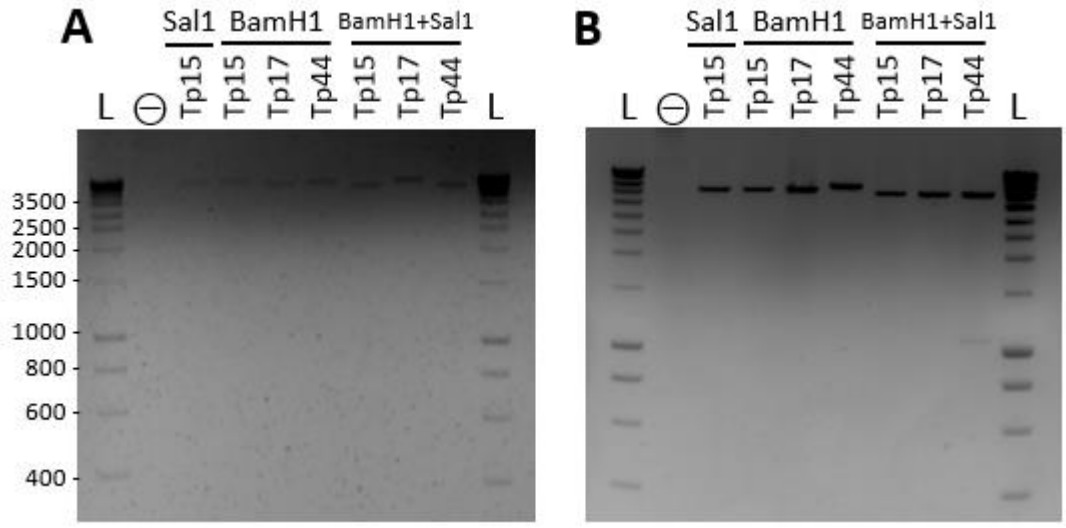
Conclusion

This validation study showed strong performance of our assay as an effective method of detecting *T. pallidum*. Each of the antigens used – Tp15, Tp17, Tp44, and Tp47 – showed "almost perfect agreement" when used with Multiplex Serology when validated against the gold standard reference tests from Fijirebio (SERODIA®) and Mikrogen (*recom*Line). Since, Multiplex Serology allows high-throughput and economical testing, the assay developed here should prove to be highly effective in large-scale epidemiological studies for *T. pallidum* as a measure of syphilis prevalence.

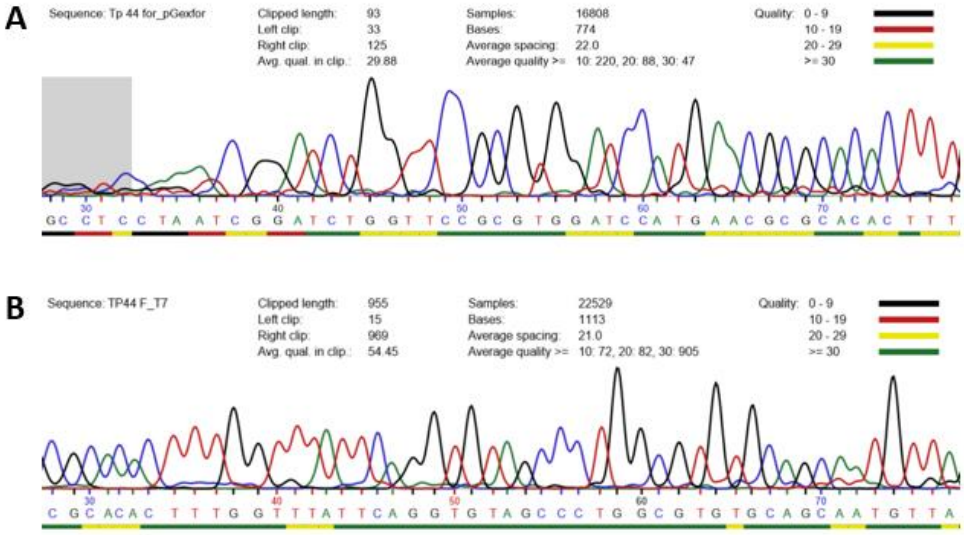
Supplementary



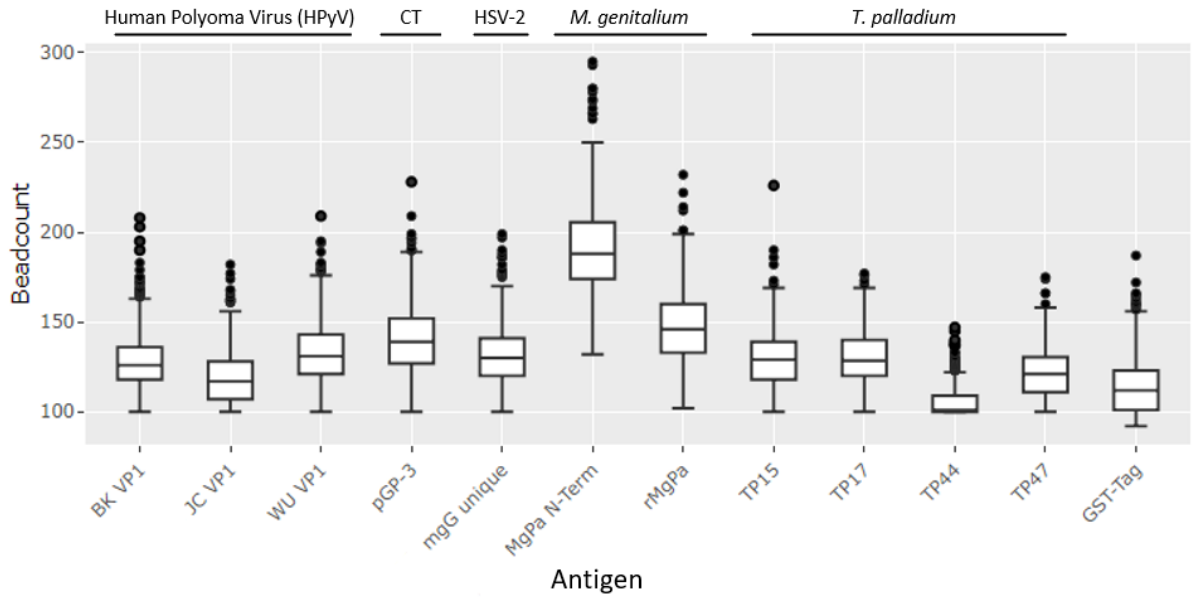
Supplementary Figure 1. Bacterial colony growth of BL21 *E. coli* after electrotransfection with pGEX4T3tag plasmids containing *T. pallidum* antigens. No growth was observed with media, nor with non-transformed *E. coli* **(A)** LB media + ampR only. Dense but distinguishable colonies were present for the transformed *E. coli* **(B)** Untransformed *E.coli* on LB + ampR. **(C)** Transformed *E.coli* on LB + ampR.



Supplementary Figure 2. Restriction digest for *T. pallidum* antigen vectors (Tp15, Tp17, Tp44, Tp47) after electrotransformation into *E. coli*. (A) 1 ng of plasmid loaded. **(B)** 10 ng of plasmid loaded. BamHI and Sal1 flank the insert on pGEX4T3tag plasmids (Figure 2). Intensity was proportional to the quantity of plasmid loaded. A single band roughly corresponding to the plasmid size (4968 bp) was present in all samples, thus indicating that digestion did not occur. A faint band appeared for 10 ng of Tp44 (1314 bp) with BamHI+Sal1 at approximately 1100 bp, but was not investigated further.



Supplementary Figure 3. Sample sequencing results for Tp44 forward strand as per Eurofins. (A) Raw plasmid sent for sequencing. Average quality extremely low (220 bp < 10 quality) and size was lower than expected (774 bp < 1047 bp) (B) PCR product sent for sequencing. Average base quality was high (919 bp > 30 quality), and low-quality bases were most dense in the end terminal region. Size was within the expected range (1113 bp ~= 1047 bp).



Supplementary Figure 4. Bead count as per Luminex flow cytometry-based analyzer for each antigen beadset. All bead counts were > 80. CT = *C. trachomatis*.

References

Agostini, H. T., Deckhut, A., Jobes, D. V., Girones, R., Schlunck, G., Prost, M. G., Frias, C., Pérez-Trallero, E., Ryschkewitsch, C. F., & Stoner, G. L. (2001). Genotypes of JC virus in East, Central and Southwest Europe. *The Journal of General Virology*, *82*(Pt 5), 1221–1331. https://doi.org/10.1099/0022-1317-82-5-1221

Alderete, J. F., & Baseman, J. B. (1980). Surface Characterization of Virulent Treponema pallidum. *Infection and Immunity*, *30*(3), 814–823.

Bremer, V, Brockmeyer, N., & Coenenberg, J. (2015). *Sexuell übertragbare Infektionen (STI) – Beratung, Diagnostik und Therapie*. https://www.awmf.org/uploads/tx_szleitlinien/059-006l_S2k_Sexuell-uebertragbare-Infektionen-Beratung-Diagnostik-Therapie-STI_2019-09.pdf

Bremer, Viviane, Dudareva-Vizule, S., Buder, S., an der Heiden, M., & Jansen, K. (2017). Sexuell übertragbare Infektionen in Deutschland. *Bundesgesundheitsblatt - Gesundheitsforschung - Gesundheitsschutz*, *60*(9), 948–957. https://doi.org/10.1007/s00103-017-2590-1

Brenner, N., Mentzer, A. J., Butt, J., Braband, K. L., Michel, A., Jeffery, K., Klenerman, P., Gärtner, B., Schnitzler, P., Hill, A., Taylor, G., Demontis, M. A., Guy, E., Hadfield, S. J., Almond, R., Allen, N., Pawlita, M., & Waterboer, T. (2019). Validation of Multiplex Serology for human hepatitis viruses B and C, human T-lymphotropic virus 1 and Toxoplasma gondii. *PLOS ONE*, *14*(1), e0210407. https://doi.org/10.1371/journal.pone.0210407

Brenner, N., Mentzer, A. J., Butt, J., Michel, A., Prager, K., Brozy, J., Weißbrich, B., Aiello, A. E., Meier, H. C. S., Breuer, J., Almond, R., Allen, N., Pawlita, M., & Waterboer, T. (2018). Validation of Multiplex Serology detecting human herpesviruses 1-5. *PLoS ONE*, *13*(12). https://doi.org/10.1371/journal.pone.0209379

Brinkman, M. B., McKevitt, M., McLoughlin, M., Perez, C., Howell, J., Weinstock, G. M., Norris, S. J., & Palzkill, T. (2006). Reactivity of antibodies from syphilis patients to a protein array representing the Treponema pallidum proteome. *Journal of Clinical Microbiology*, *44*(3), 888–891. https://doi.org/10.1128/JCM.44.3.888-891.2006

Cohen, C. E., Winston, A., Asboe, D., Boag, F., Mandalia, S., Azadian, B., & Hawkins, D. A. (2005). Increasing detection of asymptomatic syphilis in HIV patients. *Sexually Transmitted Infections*, *81*(3), 217–219. https://doi.org/10.1136/sti.2004.012187

Cooley, G. M., Mitja, O., Goodhew, B., Pillay, A., Lammie, P. J., Castro, A., Moses, P., Chen, C., Ye, T., Ballard, R., & Martin, D. L. (2016). Evaluation of Multiplex-Based Antibody Testing for Use in Large-Scale Surveillance for Yaws: A Comparative Study. *Journal of Clinical Microbiology*, *54*(5), 1321–1325. https://doi.org/10.1128/JCM.02572-15

Copen, C. (2017). Condom Use During Sexual Intercourse Among Women and Men Aged 15-44 in the United States: 2011-2015 National Survey of Family Growth. *National Health Statistics Reports*, *2017*.

DeCaprio, J. A., & Garcea, R. L. (2013). A cornucopia of human polyomaviruses. *Nature Reviews. Microbiology*, *11*(4), 264–276. https://doi.org/10.1038/nrmicro2992

Deguchi, M., Hosotsubo, H., Yamashita, N., Ohmine, T., & Asari, S. (1994). [Evaluation of gelatin particle agglutination method for detection of Treponema pallidum antibody]. *Kansenshogaku Zasshi. The Journal of the Japanese Association for Infectious Diseases*, *68*(10), 1271–1277. https://doi.org/10.11150/kansenshogakuzasshi1970.68.1271

Esposito, D., & Chatterjee, D. K. (2006). Enhancement of soluble protein expression through the use of fusion tags. *Current Opinion in Biotechnology*, *17*(4), 353–358. https://doi.org/10.1016/j.copbio.2006.06.003

Giacani, L., Molini, B. J., Kim, E. Y., Godornes, B. C., Leader, B. T., Tantalo, L. C., Centurion-Lara, A., & Lukehart, S. A. (2010). Antigenic variation in Treponema pallidum: TprK sequence diversity accumulates in response to immune pressure during experimental syphilis. *Journal of Immunology (Baltimore, Md. : 1950)*, *184*(7), 3822–3829. https://doi.org/10.4049/jimmunol.0902788

Gnanadurai, R., & Fifer, H. (2020). Mycoplasma genitalium: A Review. *Microbiology*, *166*(1), 21–29. https://doi.org/10.1099/mic.0.000830

Gossai, A., Waterboer, T., Nelson, H. H., Michel, A., Willhauck-Fleckenstein, M., Farzan, S. F., Hoen, A. G., Christensen, B. C., Kelsey, K. T., Marsit, C. J., Pawlita, M., & Karagas, M. R. (2016). Seroepidemiology of Human Polyomaviruses in a US Population. *American Journal of Epidemiology*, *183*(1), 61–69. https://doi.org/10.1093/aje/kwv155

Holmes, K. K., Levine, R., & Weaver, M. (2004). Effectiveness of condoms in preventing sexually transmitted infections. *Bulletin of the World Health Organization*, *82*(6), 454–461.

Jansen, K., Schmidt, A. J., Drewes, J., Bremer, V., & Marcus, U. (2016). Increased incidence of syphilis in men who have sex with men and risk management strategies, Germany, 2015. *Eurosurveillance*, *21*(43). https://doi.org/10.2807/1560-7917.ES.2016.21.43.30382

Kent, M. E., & Romanelli, F. (2008). Reexamining Syphilis: An Update on Epidemiology, Clinical Manifestations, and Management: *Annals of Pharmacotherapy*. https://doi.org/10.1345/aph.1K086

Korenromp, E. L., Mahiané, S. G., Nagelkerke, N., Taylor, M. M., Williams, R., Chico, R. M., Pretorius, C., Abu-Raddad, L. J., & Rowley, J. (2018). Syphilis prevalence trends in adult women in 132 countries – estimations using the Spectrum

Sexually Transmitted Infections model. *Scientific Reports*, *8*(1), 11503. https://doi.org/10.1038/s41598-018-29805-9

Krumbholz, A., Zell, R., Egerer, R., Sauerbrei, A., Helming, A., Gruhn, B., & Wutzler, P. (2006). Prevalence of BK virus subtype I in Germany. *Journal of Medical Virology*, *78*(12), 1588–1598. https://doi.org/10.1002/jmv.20743

LaFond, R. E., & Lukehart, S. A. (2006). Biological Basis for Syphilis. *Clinical Microbiology Reviews*, *19*(1), 29–49. https://doi.org/10.1128/CMR.19.1.29-49.2006

Lallemand, A., Bremer, V., Jansen, K., Nielsen, S., Muenstermann, D., Lucht, A., & Tiemann, C. (2016). Prevalence of Chlamydia trachomatis infection in women, heterosexual men and MSM visiting HIV counselling institutions in North Rhine-Westphalia, Germany—Should Chlamydia testing be scaled up? *BMC Infectious Diseases*, *16*. https://doi.org/10.1186/s12879-016-1915-2

Malhotra, M., Sood, S., Mukherjee, A., Muralidhar, S., & Bala, M. (2013). Genital Chlamydia trachomatis: An update. *The Indian Journal of Medical Research*, *138*(3), 303–316.

McGill, M. A., Edmondson, D. G., Carroll, J. A., Cook, R. G., Orkiszewski, R. S., & Norris, S. J. (2010). Characterization and Serologic Analysis of the Treponema pallidum Proteome. *Infection and Immunity*, *78*(6), 2631–2643. https://doi.org/10.1128/IAI.00173-10

Miller, P., Skov, S., & Knox, J. (1999). *How to interpret syphilis results: A manual for medical staff in remote area clinics*. https://trove.nla.gov.au/work/5617609

Neske, F., Prifert, C., Scheiner, B., Ewald, M., Schubert, J., Opitz, A., & Weissbrich, B. (2010). High prevalence of antibodies against polyomavirus WU, polyomavirus KI, and human bocavirus in German blood donors. *BMC Infectious Diseases*, *10*(1), 215. https://doi.org/10.1186/1471-2334-10-215

Pathela, P., Braunstein, S. L., Schillinger, J. A., Shepard, C., Sweeney, M., & Blank, S. (2011). Men who have sex with men have a 140-fold higher risk for newly diagnosed HIV and syphilis compared with heterosexual men in New York City. *Journal of Acquired Immune Deficiency Syndromes (1999)*, *58*(4), 408–416. https://doi.org/10.1097/QAI.0b013e318230e1ca

Pebody, R., Andrews, N., Brown, D., Gopal, R., de Melker, H., Francois, G., Gatcheva, N., Hellenbrand, W., Jokinen, S., Klavs, I., Kojouharova, M., Kortbeek, T., Kriz, B., Prosenc, K., Roubalova, K., Teocharov, P., Thierfelder, W., Valle, M., Van Damme, P., & Vranckx, R. (2004). The seroepidemiology of herpes simplex virus type 1 and 2 in Europe. *Sexually Transmitted Infections*, *80*(3), 185–191. https://doi.org/10.1136/sti.2003.005850

Peeling, R. W., Mabey, D., Kamb, M. L., Chen, X.-S., Radolf, J. D., & Benzaken, A. S. (2017). Syphilis. *Nature Reviews Disease Primers*, *3*(1), 1–21. https://doi.org/10.1038/nrdp.2017.73

Perine, P. L., Hopkins, D. R., Niemel, P. L. A., John, R. K. St., Causse, G., & Antal, G. M. (1984). *Handbook of endemic treponematoses: Yaws, endemic syphilis and pinta*. https://apps.who.int/iris/handle/10665/37178

Pierro, A., Bragaglia, L., Moroni, A., Landini, M., & Sambri, V. (2013). P2.065 Preliminary Evaluation of a Commercially Available Immunoblotting Method with Treponema Pallidum Recombinant Antigens For Serological Diagnosis of Syphilis. *Sexually Transmitted Infections*, *89*(Suppl 1), A107–A108. https://doi.org/10.1136/sextrans-2013-051184.0330

Radolf, J. D. (1996). Treponema. In S. Baron (Ed.), *Medical Microbiology* (4th ed.). University of Texas Medical Branch at Galveston. http://www.ncbi.nlm.nih.gov/books/NBK7716/

Reichert, F., Waterboer, T., Poethko-Müller, C., & Jansen, K. (2019). P611 High seroprevalence of mycoplasma genitalium in the general adult population of germany. In *Sexually Transmitted Infections* (Vol. 95, p. A270). https://doi.org/10.1136/sextrans-2019-sti.679

Schadinn, F, & Hoffmann, E. (1886). Vorlaufiger Bericht uber das Vorkommen von Spirochaeten in syphilitischen Krankheitsprodukten und bei Papillomen. *Arbeiten aus dem Kaiserlichen Gesundheitsamte*, *22*, 527–534.

Sehr, P., Zumbach, K., & Pawlita, M. (2001). A generic capture ELISA for recombinant proteins fused to glutathione S-transferase: Validation for HPV serology. *Journal of Immunological Methods*, *253*(1–2), 153–162. https://doi.org/10.1016/s0022-1759(01)00376-3

Suhandynata, R. T., Wan, L., Zhou, H., & Hollingsworth, N. M. (2016). Identification of Putative Mek1 Substrates during Meiosis in Saccharomyces cerevisiae Using Quantitative Phosphoproteomics. *PLOS ONE*, *11*(5), e0155931. https://doi.org/10.1371/journal.pone.0155931

Tate, J., & Ward, G. (2004). Interferences in Immunoassay. *The Clinical Biochemist Reviews*, *25*(2), 105–120.

Tomson, F. L., Conley, P. G., Norgard, M. V., & Hagman, K. E. (2007). Assessment of cell-surface exposure and vaccinogenic potentials of Treponema pallidum candidate outer membrane proteins. *Microbes and Infection / Institut Pasteur*, *9*(11), 1267–1275. https://doi.org/10.1016/j.micinf.2007.05.018

Trabert, B., Waterboer, T., Idahl, A., Brenner, N., Brinton, L. A., Butt, J., Coburn, S. B., Hartge, P., Hufnagel, K., Inturrisi, F., Lissowska, J., Mentzer, A., Peplonska, B., Sherman, M. E., Wills, G. S., Woodhall, S. C., Pawlita, M., & Wentzensen, N. (2019). Antibodies Against Chlamydia trachomatis and Ovarian Cancer Risk in Two Independent Populations. *JNCI: Journal of the National Cancer Institute*, *111*(2), 129–136. https://doi.org/10.1093/jnci/djy084

UniProt: A worldwide hub of protein knowledge. (2019). *Nucleic Acids Research*, *47*(D1), D506–D515. https://doi.org/10.1093/nar/gky1049

UPI00051FF3D3. (n.d.). Retrieved June 26, 2020, from https://www.uniprot.org/uniparc/UPI00051FF3D3

Viera, A. J., & Garrett, J. M. (2005). Understanding interobserver agreement: The kappa statistic. *Family Medicine*, *37*(5), 360–363.

Wald, A., & Ashley-Morrow, R. (2002). Serological Testing for Herpes Simplex Virus (HSV)–1 and HSV-2 Infection. *Clinical Infectious Diseases*, *35*(Supplement_2), S173–S182. https://doi.org/10.1086/342104

Waterboer, T., Sehr, P., Michael, K. M., Franceschi, S., Nieland, J. D., Joos, T. O., Templin, M. F., & Pawlita, M. (2005). Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clinical Chemistry*, *51*(10), 1845–1853. https://doi.org/10.1373/clinchem.2005.052381

Werner, R. N., Gaskins, M., Nast, A., & Dressler, C. (2018). Incidence of sexually transmitted infections in men who have sex with men and who are at substantial risk of HIV infection—A meta-analysis of data from trials and observational studies of HIV pre-exposure prophylaxis. *PloS One*, *13*(12), e0208107. https://doi.org/10.1371/journal.pone.0208107

World Health Organization. (2016). *WHO guidelines for the treatment of treponema pallidum (Syphilis).* http://www.ncbi.nlm.nih.gov/books/NBK384904/

Yamamoto, N., Ejima, K., & Nishiura, H. (2018). Modelling the impact of correlations between condom use and sexual contact pattern on the dynamics of sexually transmitted infections. *Theoretical Biology & Medical Modelling*, *15*. https://doi.org/10.1186/s12976-018-0078-9