Development and evaluation of an enzyme-linked immunosorbent assay for the detection of antibodies to a common urogenital derivative of Chlamydia trachomatis plasmid-encoded PGP3

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Full title: Development and Evaluation of an Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to a Common Urogenital Derivative of *Chlamydia trachomatis* Plasmid-Encoded PGP3

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Abstract

Background

Urogenital infection with *Chlamydia trachomatis* is the most commonly diagnosed sexually transmitted infection in the developed world. Accurate measurement and therefore understanding the seroprevalence of urogenital *C. trachomatis* infections requires a rigorously optimised and validated ELISA. Previous ELISAs based on the *C. trachomatis* plasmid-encoded protein, PGP3, have been described but lack standardisation and critical controls or use a less common PGP3 as the capture antigen.

Methodology/Principal Findings

A sensitive and specific indirect ELISA was developed based on recombinant PGP3 derived from a urogenital strain of *C. trachomatis*, serovar E (pSW2), using a rigorous validation protocol. Serum samples were collected from 166 genitourinary medicine (GUM) clinic patients diagnosed as positive or negative for urogenital *C. trachomatis* infection by nucleic acid amplification testing (NAATs). Overall sensitivity and specificity compared to NAATs was 68.18% and 98.0%, respectively. Sensitivities for female and male samples were 71.93% and 64.15%, respectively. Comparison of samples from these patients diagnosed positive for *C. trachomatis* by NAAT and patients diagnosed negative by NAAT revealed statistical significance ($p = <0.0001$).

Conclusions

We have developed and validated a sensitive and specific ELISA to detect anti-PGP3 antibodies as an indicator of past and current infection to *C. trachomatis* using PGP3 from a common urogenital strain. It is anticipated that this assay will be used for seroepidemiological analysis of urogenital *C. trachomatis* in populations.

Keywords: *Chlamydia trachomatis*; PGP3; ELISA; Antibody; Chlamydia infection; enzyme-linked immunosorbent assay.

Introduction

Urogenital infection with *Chlamydia trachomatis* is the most commonly diagnosed sexually transmitted infection in the developed world with an estimated 105 million new cases globally per annum (Gerbase et al. 1998; Kenyon et al. 2014). However, infections have been reported to be asymptomatic in up to 74% of cases and untreated infections have the potential to lead to more serious complications such as infertility, pelvic inflammatory disease and ectopic pregnancy (Barlow et al. 2001; Low et al. 2007). Collectively, the treatment and management of these preventable chlamydial infections and their associated comorbidities place a significant financial burden on healthcare systems. Understanding the seroprevalence of urogenital *C. trachomatis* infection in populations is essential as this would support targeted intervention and screening. However, research to measure the seroprevalence of urogenital chlamydial infection has been hindered by the lack of a universally accepted sensitive and specific serological assay.

C. trachomatis is an obligate intracellular pathogen with a biphasic developmental cycle (Ward 1983). *C. trachomatis* isolates have traditionally been classified as 'serovars' by microimmunofluorescence (MIF). These are also grouped by their biological properties into two biovars: trachoma and lymphogranuloma venereum (LGV). *C. trachomatis* isolates from the trachoma biovar comprise two separate groups: serovars A-C primarily affecting ocular tissues and serovars D-K commonly associated with sexually transmitted urogenital tract infections. The LGV biovar is much less common in the general population and includes serovars L1-L3 which are able to invade the lymphatic system. LGV is more common in some parts of the world (e.g. Africa, India, South East Asia) and in populations of men who

have sex with men (MSM) (Childs et al. 2015). Nearly all *C. trachomatis* strains carry a 7.5 kb plasmid which is never found in human isolates of *C. pneumoniae* (Campbell et al. 1987). This plasmid contains eight coding sequences (Seth-Smith et al. 2009) which contribute to chlamydial infectivity and regulation of chromosome- and plasmid-encoded genes (Carlson et al. 2008; Wang et al. 2011). PGP3 is encoded by plasmid coding sequence 5 (CDS5) and the 264 amino acid protein products polymerise to form an 84 kDa homotrimer (Galaleldeen et al. 2013). PGP3 is the only plasmid-encoded protein secreted outside the chlamydial inclusion and into the host cell cytosol (Li et al. 2008b), although neither the mechanism of protein secretion nor its primary function is known. It has recently been reported that PGP3 binds to and neutralises LL-37, an antimicrobial peptide secreted by epithelial cells and leukocytes (Hou et al. 2015). Experiments using urogenital isolates of *C. trachomatis* with plasmids where CDS5 has been deleted revealed significantly reduced bacterial burdens in the genital tracts of mice, mimicking the properties of the plasmid-free (P^-) strains (Liu et al. 2014; Ramsey et al. 2014). As most patients infected with *C. trachomatis* produce an antibody response to PGP3 (Bas et al. 2001b; Comanducci et al. 1994; Donati et al. 2009; Ghaem-Maghami et al. 2003; Goodhew et al. 2012; Wills et al. 2009), these properties make PGP3 an ideal antigen for detecting anti-*C. trachomatis* antibodies for use in ELISA formats for both diagnosis and seroprevalence.

Previous studies that used ELISA or multiplex assay to detect prior *C. trachomatis* exposure used PGP3 derived from various serovars of *C. trachomatis*, including L1 (Wills et al. 2009) and a urogenital strain, D (Bas et al. 2001a; Comanducci et al. 1994; Donati et al. 2009; Ghaem-Maghami et al. 2003; Goodhew et al. 2012). PGP3 from urogenital serovars D and E and PGP3 from the LGV serovar L1 vary by nine amino acids with different hydrophobic/hydrophilic characteristics (S1 appendix). These variations are reflected in different isoelectric points (pI) between serovars D/E and L1 (4.34 and 4.57, respectively)

which could account for structural differences affecting the sensitivity of the assay. The ELISAs developed to date lack standardisation and there is significant variation between the assay methods, which include relevant controls, origin of the PGP3 and recombinant protein purification methods.

As genital tract chlamydial infections are often asymptomatic and are known to self-clear (Corbeto et al. 2015; Morre et al. 2002), population data based upon nucleic acid detection based diagnostics which detect current infections do not represent historic exposure. Therefore, a reliable serological assay incorporating a relevant antigen is essential to gain a full understanding of the seroepidemiology of genital tract chlamydial infections in populations and to study the natural history of infections in experimental systems. Our aim was to develop and validate such a serological assay using PGP3 derived from a common urogenital tract strain of *C. trachomatis*, serovar E, which shares a 100% amino acid identity with PGP3 derived from common urogenital serovars D and F.

Materials and Methods

Cloning of PGP3 in *E. coli* expression vectors

Coding sequence 5 (CDS5) was amplified by PCR from recombinant plasmid pSP73-SW2 (Seth-Smith et al. 2009) (*C. trachomatis* pSW2; EMBL: **FM865439**) with Phusion Flash PCR Master-Mix (ThermoFisher Scientific) according to the manufacturer's protocol using custom primers [F] 5'-gcagcaGGATCCatgggaaattctggtttttatttgt-3' and [R] 5'-gcagcaCTCGAGattgtttaagcgttgtttgagg-3'. Amplified PCR constructs were digested with XhoI and BamHI (New England Biolabs) and ligated into the expression vector pGEX-4T1/6P-1 (GE Healthcare Life Sciences) using T4 DNA ligase (Promega, Southampton) to produce recombinant N-terminal glutathione s-transferase fusion tag protein expression vectors according to manufacturers' instructions. These plasmids were sequence verified

and then transformed into *Escherichia coli* BL21 (DE3) pLysS (Hoffman et al. 1995) for protein expression.

Recombinant protein expression and purification

E. coli BL21 (DE3) pLysS carrying expression vectors were grown to an optical density of ~0.6 in Super Optimal Broth (SOB) media (0.5% yeast extract, 0.05% NaCl, 2% tryptone, 2.6 mM KCl, 10 mM $MgCl_2$) and induced for 4 hours at 37°C with 1 mM Isopropyl β-D-thiogalactopyranoside (Thermo Fisher Scientific). Pellets were freeze-thawed and lysed using BugBuster Master Mix (Merck Millipore) and cOmplete Protease Inhibitor Cocktail tablets (Roche Applied Science) according to the manufacturers' instructions. Lysed cells were centrifuged and protein-containing supernatant fractions were incubated with glutathione sepharose 4B beads (GE Healthcare Life Sciences) for 1 hour at room temperature. Beads were washed twice in PBS using disposable chromatography columns. Recombinant GST-PGP3 (54 kDa) fusion proteins expressed using the pGEX-4T-1 vector were washed once more with PBS and bound GST-tagged protein was eluted using buffer A (50 mM reduced glutathione (Sigma-Aldrich), 50 mM Tris-HCl [pH 8]). Empty vectors of pGEX-4T-1 and pGEX-6P-1 were also induced using the same protocol to produce the GST fusion tag (26 kDa). Recombinant GST-PGP3 fusion proteins expressed using the pGEX-6P-1 vector were washed twice more in buffer B (50 mM Tris, 150 mM NaCl, 5 mM DTT [pH 7]). Recombinant PGP3 protein was cleaved from bound GST using 48 units of GST-tagged Prescission Protease (46 kDa) (GE Healthcare Life Sciences) in buffer B and incubated at room temperature for 4 hours then at 4°C for 16 hours. Pooled fractions were dialysed against PBS overnight at 4°C using CelluSep T1 cellulose membrane (MWCO: 3,500) (Membrane Filtration Products, Inc). Protein concentrations were determined using the Pierce 660 nm protein assay reagent and bovine serum albumin standards

(ThermoFisher Scientific) according to the manufacturer's protocol. Aliquots of purified proteins were analysed against bovine serum albumin standards using SDS-PAGE.

Polyacrylamide gel electrophoresis

10 µl aliquots (1 µg) of purified fractions were denatured in sodium dodecyl sulphate (SDS) buffer (1.25% β-mercaptoethanol (BME), 2% SDS, 0.01% bromophenol blue, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8]) for 5 minutes at 100°C and analysed by SDS polyacrylamide gel electrophoresis on 12% gels.

Serum samples

Rabbit polyclonal antiserum to cleaved purified PGP3 was prepared as previously described using four fortnightly immunisations of 50 µg protein (Watson et al. 1994). Anonymised and unlinked sera from patients who attended Southampton genitourinary medicine (GUM) clinic for sexual health screening were collected. These patients also provided swab or urine samples for chlamydial diagnosis by NAAT (APTIMA Combo 2 for CT/NG Assay, Hologic, Crawley, UK). Details included the chlamydial infection status as diagnosed by NAAT (PHE Regional Microbiology Laboratory, Southampton) at the time of serum collection, patient age and patient sex. 61 samples were from *C. trachomatis* NAAT positive females, 56 from *C. trachomatis* NAAT positive males and 62 were *C. trachomatis* NAAT negative patients of mixed gender. Patient sera were stored at -20 °C until ready to use. Most samples had undergone 1 freeze-thaw cycle. Approximately 20 samples used in initial assay and western blotting optimisation experiments had undergone more than 5 freeze-thaw cycles.

Ethics

The work with human sera was approved by NHS Research Ethics Committee (Chlamydial antibodies in infection [16/NW/0346]) and this project was approved by the University of

Southampton Ethics and Research Governance Committee ID 19708 'Detection of chlamydial antibodies.' Patient serum samples are submitted to the PHE Regional Microbiology Laboratory, Southampton, for routine diagnostic testing. Following completion of their analysis and reporting of results, discarded samples were collected. Consent was waived as permitted by the NREC as these samples were unlinked and anonymised to protect patient confidentiality. The only details retained were patient age at the time of sample collection (but not date of birth) and patient sex to allow us to separate results based on sex and diagnosed *C. trachomatis* infection.

Indirect ELISA

Blocking conditions, blocking agents, concentrations of patient sera and secondary antibody and choice of ELISA plates were initially optimised in a series of experiments with positive control antibodies, anonymised *C. trachomatis* NAAT negative patient sera and *C. trachomatis* NAAT positive patient sera. For sensitivity and specificity calculations, Medisorp flat-bottom 96-well plates (Nunc, ThermoFisher Scientific) were coated at 500 ng/well of GST-PGP3 and cleaved PGP3. GST was used to coat plates at 250 ng/well to match the fusion tag:recombinant protein ratio. Plates were washed three times in 0.1% PBS Tween 20 (PBS-T) and blocked in 1% Hammarsten grade sodium caseinate (Affymetrix) in PBS-T (200 µl/well) for two hours at 37°C. Plates were washed three times in PBS-T patient sera with a known *C. trachomatis* NAAT status were added at 1:100 (50 µl/well) in duplicate for 1 hour at 37°C. Plates were washed six times in PBS-T and incubated with HRP-labelled goat anti-human/mouse IgG (Bio-Rad) diluted 1:8000 (100 µl/well) for 1 hour at 37°C. All sera and antibody dilutions were prepared in 1% Hammarsten grade sodium caseinate in PBS-T. Plates were washed for a final six times before adding 100µl/well of ready-made 3,3',5,5'-tetramethylbenzidine (TMB) solution (eBioscience) for 10 minutes. The reaction

was stopped with 50µl/well 2M H_2SO_4 and absorbance was read at $O.D_{450}$ using a BioRad iMark microplate absorbance reader. Background absorbances of each patient was corrected by subtracting the average absorbance of each duplicate well containing serum, blocking, conjugate and TMB but no antigen, from the average absorbance of duplicate wells containing antigen, serum, blocking, conjugate and TMB.

Western blotting of recombinant PGP3 and GST

To observe the monomeric structure of GST-PGP3, 10 µl aliquots (1 µg) were treated with SDS, BME and heated at 100°C for 5 minutes, as described above. To observe trimeric structures, 10 µl aliquots were treated with native loading buffer (no SDS or BME) and were not heat treated. Proteins were then separated by SDS-PAGE and transferred to a polyvinylidene diflouride (PVDF) Immobilon membrane (EMD Millipore) in Pierce Fast Semi-Dry Buffer (ThermoFisher Scientific) using a Pierce Fast Semi-Dry Blotter. After blocking in 10% skimmed milk solution (Marvel, UK) in PBS-T for 1 hour at room temperature (RT), membranes were washed three times and incubated with human (1:200) primary antibodies for one hour at RT. Membranes were washed and incubated with secondary antibodies diluted 1:2000 for 1 hour at RT (HRP-labelled goat anti-rabbit/human/mouse IgG (Bio-Rad). Membranes were washed three times and visualised using Pierce enhanced chemiluminescence (ECL) system western blotting substrate (ThermoFisher Scientific). Positive control antibodies included rabbit polyclonal anti-PGP3 IgG diluted 1:2000 and mouse monoclonal anti-GST IgG (ThermoFisher Scientific) diluted 1:1000.

Statistical analyses

GraphPad Prism version 6.0 (GraphPad Software Inc., USA) was used to calculate sensitivities and specificities of assays using Receiver Operating Characteristic (ROC) curve

analyses and intra- and interassay variation of assays stating means, standard deviations, standard errors and coefficient of variation (CV). Results were considered to be statistically significant if p = <0.05. Serum details and background-corrected OD_{450} results obtained from ELISAs were organised and calculated using Microsoft Excel from Microsoft Office 2010.

Results and Discussion

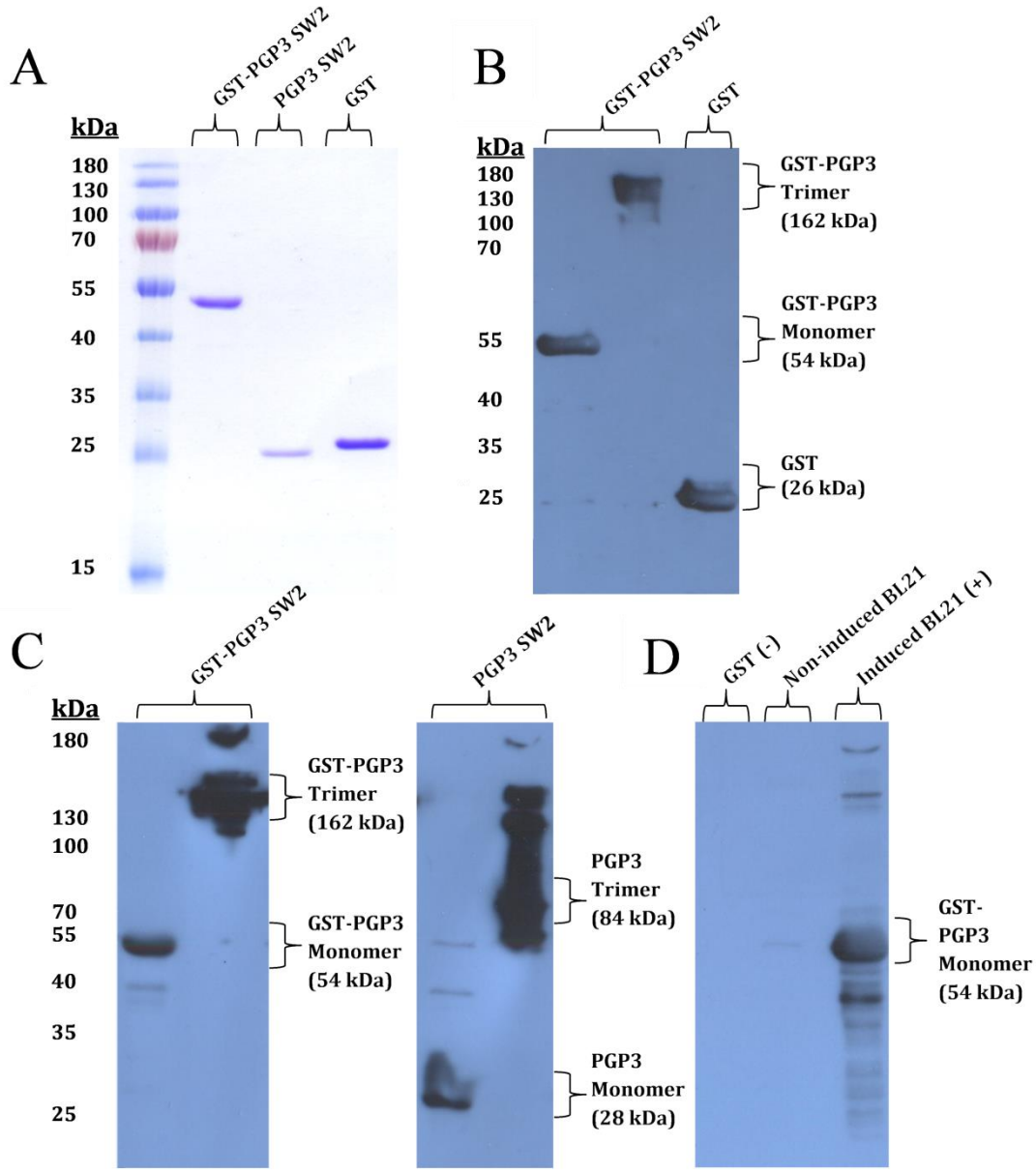
PGP3 derived from the urogenital strain of *C. trachomatis*, serovar E, is the most appropriate antigen for seroepidemiological analysis of urogenital *C. trachomatis* in populations as it is one of the most common urogenital genital tract strains in the developed world (Millman et al. 2004; Spaargaren et al. 2004). The DNA sequence is highly conserved amongst urogenital isolates; PGP3 derived from the common urogenital serovars D (pCHL1; Genbank: NC_001372), E (pSW2; EMBL: 865439) and F (pSW4; EMBL: 865441) share a 100% amino acid identity.

Configuration, expression and purification of recombinant PGP3

Recombinant GST-PGP3 can be easily and efficiently expressed, purified and dialysed against PBS in less than 5 days with typical yields of 600 µg recombinant protein per 250 ml cell culture which can easily be up-scaled. All reagents are available commercially making transfer of the technology between laboratories straightforward and reproducible. The purity of recombinant GST-PGP3, PGP3 and GST used in this study can be seen in Figure 1A. Monomers and trimers of GST-PGP3 and PGP3 were observed using Western blot (Figure 1B and 1C). Monomeric structures of GST-PGP3 (54 kDa) and PGP3 (28 kDa) were observed by boiling in 2% SDS and 1.25% BME. To observe trimeric structures, GST-PGP3 (162 kDa) and PGP3 (84 kDa) were left untreated (no added SDS or BME and no heat treatment). Purified PGP3 that had been cleaved from the GST fusion tag was used to produce rabbit polyclonal anti-PGP3 antibody. Using Western blot, rabbit polyclonal anti-

PGP3 antibody was blotted against GST and GST-PGP3 expressed in whole cells of BL21 (DE3) pLysS *E. coli* (Figure 1D). No reaction to GST or any non-specific products were observed.

Figure 1. Analysis of recombinant PGP3 expression and trimerisation by polyacrylamide gel electrophoresis. Recombinant PGP3 derived from *C. trachomatis* pSW2 (serovar E) expressed as a fusion protein with an N-terminal glutathione s-transferase tag was purified from an *E. coli* expression system in which the tag was retained (GST-PGP3) or subsequently cleaved (PGP3). **[A]** Samples of purified protein were denatured in SDS buffer (2% SDS, 1.25% β-mercaptoethanol (BME)), heated to 100°C for 5 minutes, analysed using a 12% SDS gel and visualised using Coomassie brilliant blue staining. **[B]** GST-PGP3 trimerisation. Samples of GST-PGP3 were treated with SDS buffer (as described in [A]) or were left untreated to observe monomeric and trimeric GST-PGP3, respectively. Samples were analysed by SDS-PAGE, transferred to a PVDF membrane, incubated with a mouse monoclonal antibody to GST (mAb anti-GST) and goat anti-mouse IgG-HRP and then visualised using a chemiluminescence kit. **[C]** Monomers and trimers of GST-PGP3 and PGP3 were visualised as described in [A] and [B] using a rabbit polyclonal antibody to PGP3 (pAb anti-PGP3). pAb rabbit anti-PGP3 was produced by immunisation using cleaved PGP3 derived from *C. trachomatis* pSW2 (serovar E). **[D]** Rabbit pAb anti-PGP3 was blotted against GST-PGP3 expressed in whole cells of BL21 (DE3) pLysS *E. coli* induced for 4 hours. Rabbit pAb anti-PGP3 did not react with the GST negative control (-). A weak reaction to GST-PGP3 in non-induced *E. coli* was observed.



Design and optimisation of the GST-PGP3 SW2 ELISA

Blocking conditions, blocking agents, antigen concentration, concentrations of patient sera and secondary antibody and choice of ELISA plates were optimised in a series of experiments. Mouse monoclonal anti-GST antibody (Invitrogen, ThermoFisher Scientific), rabbit polyclonal anti-PGP3 antibody and anonymised sera from patients who had tested positive or negative to *C. trachomatis* by NAAT were used for assay optimisation. During these experiments we discovered that each human serum sample resulted in different background readings when assayed in blocked non-antigen coated wells. Therefore, we chose

to correct the absorbance readings for background by subtracting the absorbance reading of each well containing serum but no antigen from the absorbance reading of wells containing antigen and serum. This method of background correction has previously been used in another PGP3 assay (Bas et al. 2001b).

GST (Figure 1) is a suitable negative control antigen as it is expressed and purified using the same vectors and protocol as the GST-PGP3 fusion proteins and it can be used to assess false-positive reaction from sera with the fusion tag. Therefore, we considered it necessary to assay all sera against the GST tag as part of the protocol. GST expressed from pGEX expression vectors (GE Healthcare) is derived from *Schistosoma japonicum*, one of the main causative agents of schistosomiasis in rural regions of Asia, particularly China (Li et al. 2000). Sera collected from patients in the United Kingdom are unlikely to contain antibodies to GST. This GST antigen was used to coat plates at a concentration to match the fusion tag:polypeptide ratio of the GST-PGP3. Inclusion of GST is an additional internal and rigorous control since no reaction to GST paired with any reaction to GST-PGP3 in an ELISA or western blot would be solely due to the presence of anti-PGP3 antibodies in patient sera. The use of GST as a negative control in PGP3 serological assays and western blots has also been demonstrated in other studies (Chen et al. 2010; Li et al. 2008a; Wang et al. 2010).

Sensitivity and specificity of the GST-PGP3 ELISA

Purified GST-PGP3 from *C. trachomatis* serovar E (pSW2) was used to perform an ELISA using sera collected from 166 GUM clinic patients diagnosed as positive or negative for *C. trachomatis* by nucleic acid amplification testing (NAAT). 57 were from females tested positive for *C. trachomatis*, 53 from males tested positive for *C. trachomatis* and 56 patients of mixed-sex tested negative for *C. trachomatis*. All sera were assayed in duplicate against GST-PGP3 and were also assayed against the GST negative control antigen. Data were

grouped according to the patient sex and NAAT status (Figure 2). Receiver operator characteristics (ROC) were produced for the GST-PGP3 SW2 indirect ELISA. At 98% specificity, the GST-PGP3 SW2 ELISA (Table 1) was 71.93% sensitive for females (OD_{450} cut-off: 0.652) and 64.15% for males (OD_{450} cut-off: 0.665). Comparison of female and male groups of patients who had tested positive for *C. trachomatis* by NAAT with patients who tested negative for *C. trachomatis* by NAAT revealed statistical significance (one-way ANOVA Tukey multiple comparison test: p = <0.0001). All patient sera returned a background-corrected OD_{450} value against GST below 0.481 (Figure 2). Six serum samples from patients with a negative *C. trachomatis* diagnosis by NAAT returned OD_{450} values above 0.98 in our GST-PGP3 SW2 ELISA (Table 2). However, these sera were assayed against GST and background-corrected OD_{450} values ranged -0.066 to 0.127. These samples were subsequently assayed by western blot and reacted with GST-PGP3 (Figure 3 and appendix S2), indicating possible past exposure to *C. trachomatis* as these patients are likely to have been sexually active.

Figure 2. Antibody responses to GST-PGP3 by indirect ELISA using patient sera. Graphs show background-corrected mean OD_{450} values of sera collected from patients who had been tested for *C. trachomatis* by NAAT. Sera were assayed in duplicate at 1:100 against N-terminal glutathione s-transferase-tagged PGP3 derived from *C. trachomatis* pSW2 (serovar E). All sera were also assayed against GST as a negative control. Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean and standard deviation bars are shown. Dotted lines represent the selected cut-off points as calculated by receiver operator characteristic. * = statistical significance (p = <0.0001) between group marked by * and patients tested negative for *C. trachomatis* by NAAT (NAAT -ve). [GST-PGP3 and GST ELISAs: NAAT +ve females = 57; NAAT +ve males = 53; NAAT –ve mixed = 50].

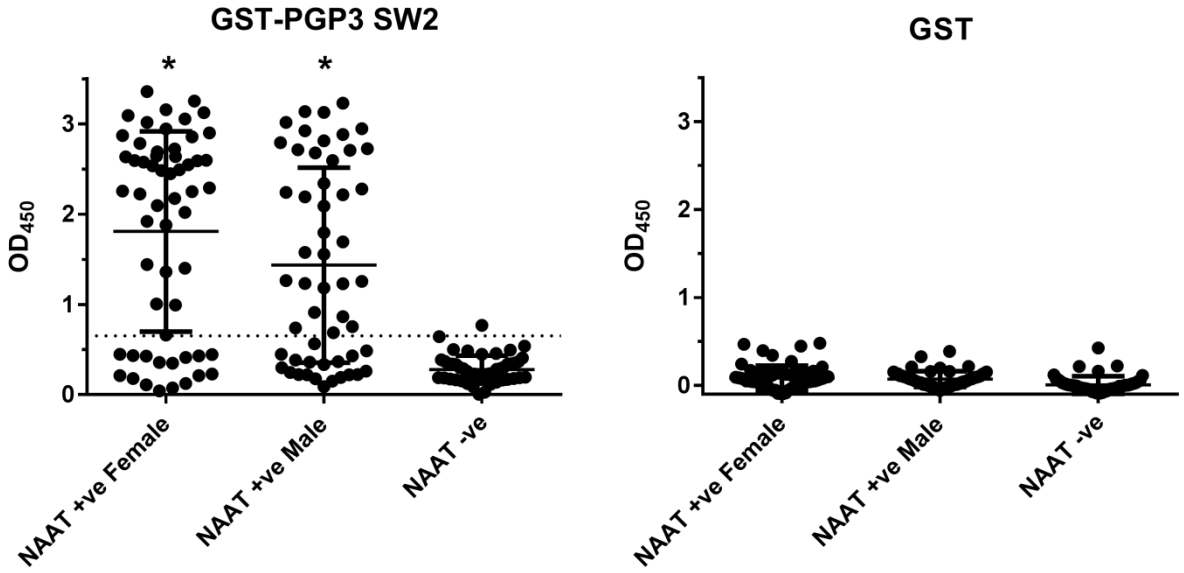


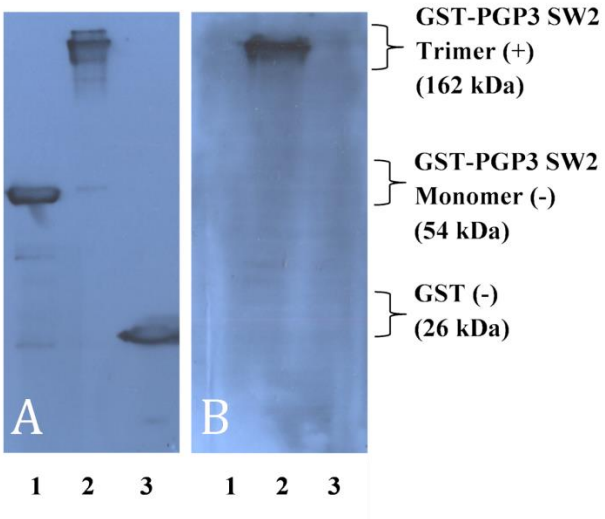
Table 1. Sensitivity and specificity of the GST-PGP3 SW2 ELISA. GraphPad Prism version 6.0 was used to calculate sensitivities and specificities of assays using Receiver Operating Characteristic (ROC) curve analyses. Results were considered to be statistically significant if p = <0.05.

Patient group	Cut off OD_{450}	ROC Area	P value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
Females	0.652	0.864	<0.0001	71.93 (58.46-83.03)	98.00 (89.35-99.95)
Males	0.665	0.840	<0.0001	64.15 (49.80-76.86)	98.00 (89.35-99.95)
Both	0.652	0.852	<0.0001	68.18 (58.62-76.74)	98.00 (89.35-99.95)

Table 2. Details of sera from patients who were diagnosed negative for *C. trachomatis* by NAAT at the time of sera collection but had high OD_{450} values in the GST-PGP3 ELISA. Sera were assayed at 1:100 against 500 ng/well GST-PGP3 SW2 and 250 ng/well GST. OD_{450} values shown are mean absorbance of two duplicate background-corrected values.

Patient No.	GST-PGP3 SW2 ELISA	GST ELISA (-ve control)
9	2.31	-0.029
11	1.85	-0.004
28	1.99	-0.066
46	2.75	0.066
58	2.26	0.011
61	2.90	0.127

Figure 3. Human antibody recognition of PGP3 in a western blot using sera from a patient who tested negative for *C. trachomatis* by NAAT. GST-PGP3 derived from *C. trachomatis* pSW2 (serovar E) was treated (lane 1: 2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 minutes) or untreated (lane 2), analysed by SDS-PAGE on a 12% SDS gel and transferred to PVDF membrane. A mouse monoclonal antibody to GST was included as a positive control **(panel A)**. Patient serum was added at 1:200 **(panel B)** and antibodies detected using goat anti-human HRP-conjugated secondary antibody (1:2000) and a chemiluminescence kit. GST was included as a negative control and was SDS and heat-treated (lane 3). All 6 sera that returned an OD_{450} value above 0.98 in an indirect GST-PGP3 SW2 ELISA were blotted against monomeric and trimeric GST-PGP3 SW2 and GST negative control antigen. All reacted with trimeric GST-PGP3 SW2 (one example is shown). None reacted with monomeric GST-PGP3 SW2 or GST negative control antigen.



Wills *et al.* (Wills et al. 2009) evaluated their ELISA (based on PGP3 antigen derived from the L1 serovar) using well-characterised sera from 356 patients of mixed-sex who tested positive for *C. trachomatis* by NAAT (serum collected one month after diagnosis). Their negative controls included sera from over 700 children aged 2-13-years-old that were presumed negative for any past exposure to *C. trachomatis*, although 25 of these were positive for anti-*C. trachomatis* antibodies by microimmunofluorescence assay. The overall sensitivity and specificity for their ELISA was 57.9% and 97.6%, respectively. Sensitivity for female samples was higher at 73.8%, 1.9% higher than female samples in our GST-PGP3

SW2 ELISA. However, the overall sensitivity of our GST-PGP3 SW2 ELISA was 10.3% higher. Furthermore, sensitivity for male samples in our ELISA was higher at 64.1%, a difference of 19.9%. Using most of the same serum cohort as Wills *et al.* (Wills et al. 2009), Horner *et al.* (2016) (Horner et al. 2016) very recently described a double-antigen PGP3 ELISA with reported improved sensitivities of 82.9% and 54.4% for female and male samples, respectively, at 97.8% specificity. This report describes an assay that is 10.97% more sensitive for female serum samples than our assay. However, our assay is 9.75% more sensitive for male serum samples. Furthermore, and most importantly, our assay GST-PGP3 SW2 ELISA allows the use of recombinant GST as a negative control antigen to assess false positive reactivity from the sera. Interestingly, although not statistically significant, our GST-PGP3 SW2 ELISA was 7.78% more sensitive for female samples than male samples. This correlates with both findings by Wills *et al.* (Wills et al. 2009) and Horner *et al.* (Horner et al. 2016) in which sensitivities for female samples were higher than males in their PGP3 ELISA and three *C. trachomatis* commercial ELISAs: the Medac pELISA Plus, the Savyon SeroCT-IgG ELISA, and the Ani Labsystems IgG enzyme immunoassay. Previous studies have found that oestrogen *in vitro* enhances the attachment of *C. trachomatis* to human endometrial epithelial cells (Maslow et al. 1988) and a higher chlamydial burden is associated with increased levels of anti-*C. trachomatis* antibodies (Agrawal et al. 2009). This may account for the higher sensitivities seen in ELISAs using serum samples from females.

Control sera used for ELISA validation

At least seven serological assays (Bas et al. 2001b; Comanducci et al. 1994; Donati et al. 2009; Ghaem-Maghami et al. 2003; Goodhew et al. 2012; Horner et al. 2016; Wills et al. 2009) using PGP3 have been described and two have reported sensitivities and specificities as high as 95.1% (Ghaem-Maghami et al. 2003) and 100% (Comanducci et al. 1994), respectively. Variation in the number of positive and negative serum controls, the source of

serum controls, methods of serum characterisation, assay type, background correction protocols, protein purification method and PGP3 serovar derivation have resulted in ranges of reported sensitivities and specificities which have made the assays incomparable. Some studies separated positive and negative serum cohorts based on *C. trachomatis* diagnosis by NAAT (Bas et al. 2001b; Ghaem-Maghami et al. 2003; Goodhew et al. 2012; Horner et al. 2016; Wills et al. 2009), presence of symptoms (Bas et al. 2001b) or isolation of chlamydia by culture (Donati et al. 2009). Bas *et al.* (2001) (Bas et al. 2001b) also obtained their negative serum cohorts from 'healthy donors' but did not confirm the absence or presence of *C. trachomatis* using any other methods. Other studies have separated their cohorts based on the presence or absence of anti-*C. trachomatis* antibodies as confirmed by MIF (Comanducci et al. 1994). Since we have developed an ELISA to detect anti-PGP3 antibodies for the seroepidemiological analysis of chlamydia, MIF is not an appropriate alternative method of serum characterisation for assay validation because MIF is based on antibody reaction with chlamydia and this would bias the analysis of pre-selected serum controls. To validate our ELISAs, we selected sera from male and female patients with a recent diagnosis of *C. trachomatis* by NAAT and compared their PGP3 ELISA data with ELISA data using sera from mixed-sex patients with a negative *C. trachomatis* diagnosis. It should be noted that patients with a negative NAAT diagnosis may have previously been exposed to *C. trachomatis*. As our sera were collected from patients at the time of *C. trachomatis* diagnosis by NAAT, not all patients with a primary infection may have seroconverted before serum collection. Furthermore, although our negative control serum samples were taken from patients with a negative NAAT diagnosis for *C. trachomatis*, these patients were nonetheless presumably sexually active (as they proactively attended the GUM clinic) and serum samples may have a potential to contain anti-PGP3 antibodies from previous *C. trachomatis* exposure. This was the case when 6 serum samples from patients with a negative *C. trachomatis*

diagnosis by NAAT returned OD_{450} values above 0.98 in our GST-PGP3 ELISAs, as described above (Table 2).

Inter- and intra-assay reproducibility of the GST-PGP3 SW2 ELISA

We analysed the intra- and inter-assay variation of the GST-PGP3 SW2 ELISA. For these calculations, we selected three sera that were negative and three that were positive for anti-PGP3 antibodies, as determined from previous ELISAs and western blotting experiments. These sera were used to determine the intra- and inter-assay and inter-operator reproducibility of the GST-PGP3 SW2 ELISA. Sera were assayed at 1:100. Mouse anti-GST monoclonal (Invitrogen, ThermoFisher Scientific) and rabbit anti-PGP3 polyclonal antibodies were also selected as positive controls and were each assayed at 1:10,000. Each serum was assayed in duplicate on each plate (intra-) on each independent assay (inter-assay variation) for a total of twenty independent assay runs using four operators, including two project independent operators and one group-independent operator. Mean OD_{450} values, standard deviations and coefficient of variation for each control were calculated using background corrected OD_{450} values on GraphPad Prism 6.0. The standard deviations of all positive and negative controls across twenty independent assays ranged from 0.032 to 0.195 (Table 3). Coefficient of variation (CV) ranged between 5.85 to 12.6% for inter-assay variation and between 2.64 to 7.14% for intra-assay variation for positive control sera and antibodies.

The GST-PGP3 SW2 indirect ELISA was repeated twice using all the patient sera and a different protein batch of GST-PGP3 SW2 but excluding the serum samples from patients diagnosed as negative for *C. trachomatis* by NAAT that reacted to PGP3 by ELISA and Western blot, as described above. A further two samples were excluded as there was insufficient sera. Overall specificity and sensitivity were 96.43% (95% CI; 87.69-99.56) and 68.70% (95% CI; 59.38-77.02), respectively.

Table 3. Inter- and intra-assay variation of the GST-PGP3 SW2 indirect ELISA. Six sera from patients (assayed in duplicate at 1:100) and anti-GST and anti-PGP3 antibodies (1:10,000) assayed against GST-PGP3 SW2 antigen. Data were calculated based on means calculated from background-corrected duplicate OD_{450} values from two plates over twenty independent ELISAs (n=20). Positive control sera and antibodies are in **bold**. **[A]** inter-assay variation; **[B]** intra-assay variation.

A

Serum No./ Control	204	205	234	215	216	217	Anti-PGP3	Anti-GST
Mean OD_{450}	**2.927**	**1.357**	**0.924**	0.280	0.108	0.042	**3.011**	**1.547**
Min OD_{450}	2.513	1.103	0.738	0.079	0.047	-0.045	2.641	1.286
Max OD_{450}	3.165	1.696	1.161	0.363	0.156	0.122	3.396	1.901
SD	**0.175**	**0.156**	**0.102**	0.085	0.032	0.04	**0.176**	**0.195**
SEM	0.039	0.035	0.023	0.019	0.007	0.009	0.039	0.044
CV (%)	**5.96**	**11.50**	**11.00**	30.31	29.58	95.88	**5.85**	**12.60**

B

Serum No./ Control	Mean OD_{450}	Mean SD	Mean CV (%)
204	**2.927**	**0.076**	**2.64**
205	**1.357**	**0.092**	**6.75**
234	**0.923**	**0.056**	**6.11**
215	0.280	0.044	27.3
216	0.108	0.026	25.5
217	0.042	0.020	41.1
Anti-PGP3	**3.011**	**0.172**	**5.71**
Anti-GST	**1.547**	**0.113**	**7.14**

Taken together these results show we have developed a robust, reliable and accurately reproducible serological assay using PGP3 in the format of a fusion protein with GST. The simple and straightforward protein purification protocols along with the incorporation of background blocking for human sera makes this a reliable method that can be deployed in any basic serology/immunology laboratory.

Conclusions

- We have developed a sensitive and specific serological assay to detect anti-PGP3 antibodies as an indicator of past and current infection to *C. trachomatis* using a common urogenital derivative of PGP3.
- The reproducibility of the GST-PGP3 SW2 ELISA was demonstrated by using well characterised controls and several operators to calculate inter- and intra-assay variation.
- GST expressed and purified using the same protocol as GST-PGP3 fusion proteins is a rigorous and relevant internal control used to assess potential false-positive background from sera in our PGP3 ELISAs.
- The overall sensitivity and specificity of the ELISA using GST-PGP3 derived from urogenital *C. trachomatis* serovar E (pSW2) was 68.18% and 98.0%, respectively.
- At 98% specificity, individual sensitivities for human female and male samples were 71.93% and 64.15%, respectively.

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Supporting Information

S1 appendix. **Amino acid alignment of PGP3 sequences from *C. trachomatis* serovars L1 (pLGV440) and E (pSW2).**

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MGNSGFYLYNTQNCVFADNIKVGQMTEPLKDQQIILGTTSTPVAAKMTASDGISLTVSNN   60 LGV440
MGNSGFYLYNTENCVFADNIKVGQMTEPLKDQQIILGTTSTPVAAKMTASDGISLTVSNN   60 SW2

PSTNASITIGLDAEKAYQLILEKLGDQILGGIADTIVDSTVQDILDKITTDPSLGLLKAF   120 LGV440
SSTNASITIGLDAEKAYQLILEKLGDQILDGIADTIVDSTVQDILDKIKTDPSLGLLKAF   120 SW2

NNFPITNKIQCNGLFTPRNIETLLGGTEIGKFTVTPKSSGSMFLVSADIIASRMEGGVVL   180 LGV440
NNFPITNKIQCNGLFTPSNIETLLGGTEIGKFTVTPKSSGSMFLVSADIIASRMEGGVVL   180 SW2

ALVREGDSKPYAISYGYSSGVPNLCSLRTRIINTGLTPTTYSLRVGGLESGVVWVNALSN   240 LGV440
ALVREGDSKPCAISYGYSSGIPNLCSLRTSITNTGLTPTTYSLRVGGLESGVVWVNALSN   240 SW2

GNDILGITNTSNVSFLEVIPQTNA  264 LGV440
GNDILGITNTSNVSFLEVIPQTNA  264 SW2
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S2 appendix. Human antibody recognition of PGP3 in a western blot using sera from patients who tested negative for *C. trachomatis* by NAAT. GST-PGP3 derived from *C. trachomatis* pSW2 (serovar E) was treated (lane 1: 2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 minutes) or untreated (lane 2), analysed by SDS-PAGE on a 12% SDS gel and transferred to PVDF membrane. A mouse monoclonal antibody to GST was included as a positive control **(panel A)**. Patient serum was added at 1:200 **(panels B-F)** and antibodies

detected using goat anti-human HRP-conjugated secondary antibody (1:2000) and a chemiluminescence kit. GST was included as a negative control and was SDS and heat-treated (lane 3). All 6 sera that returned an OD_{450} value above 0.98 in an indirect GST-PGP3 SW2 ELISA were blotted against monomeric and trimeric GST-PGP3 SW2 and GST negative control antigen. All reacted with trimeric GST-PGP3 SW2 (serum no. 58 is not shown as this returned a very high background). None reacted with monomeric GST-PGP3 SW2 or GST negative control antigen.