THE SEROEPIDEMIOLOGY OF *CHLAMYDIA TRACHOMATIS* IN 13-15-YEAR-OLDS IN ENGLAND

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ABSTRACT

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THE SEROEPIDEMIOLOGY OF *CHLAMYDIA TRACHOMATIS* IN 13-15-YEAR-OLDS IN ENGLAND: by Catherine E. Winstanley

Urogenital infection with *Chlamydia trachomatis* is the most commonly diagnosed sexually transmitted infection in the developed world. No systematic investigation of the seroprevalence of *C. trachomatis* in the under 16s population in England has been conducted. This is despite evidence of chlamydial diagnoses and increasingly earlier sexual debut. As the National Chlamydia Screening Programme only recruits patients aged 15-24-years-old for chlamydial screening, seroprevalence data will provide insight into the need for screening younger patients. However, 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 commonly derived PGP3 as the capture antigen. The aim of this study was to develop a sensitive and specific indirect ELISA based on recombinant PGP3 derived from a urogenital strain of *C. trachomatis*, serovar E (pSW2), using a rigorous validation protocol. The next aim was then to apply this ELISA to detect anti-PGP3 antibodies in a significant number of patient sera collected from patients under 16-years-old from England to determine the seroprevalence of *C. trachomatis* in this population.

To evaluate the ELISA, serum samples were collected from 166 male and female genitourinary medicine clinic patients diagnosed as positive or negative for urogenital *C. trachomatis* infection by nucleic acid amplification testing. Overall sensitivity and specificity compared to nucleic acid amplification testing 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 nucleic acid amplification testing and patients diagnosed negative by nucleic acid amplification testing revealed statistical significance ($p = <0.0001$). This ELISA was further validated by demonstrating that sera from mice urogenitally infected with *C. trachomatis* produce measureable responses to recombinant PGP3. In contrast to previous reports, it has also been shown that human antibody recognition of PGP3 is not solely dependent on its homotrimeric conformation.

To determine the seroprevalence of *C. trachomatis* in the under 16s population in England, 2119 serum samples received from the Seroepidemiology Unit were assayed. Overall seroprevalence in 13-, 14- and 15-year-olds from all combined serum sources was 6.87%, 6.70% and 10.47%, respectively. The seroprevalence of antibodies to PGP3 in serum samples collected from male and female 13-, 14- and 15-year-olds not sourced from GUM clinics was 4.66%, 4.37% and 10.78%, respectively. The overall seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from an unknown or unrecorded source was 7.10%, 6.26% and 6.75%, respectively. Finally, the seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from GUM clinics was 18.52%, 18.18% and 24.32%, respectively.

In conclusion, for the first time, the seroprevalence rate of anti-PGP3 antibodies within serum samples collected from 13-15-year-old children in England has been determined using a validated sensitive and specific ELISA based on recombinant PGP3 derived from *C. trachomatis*, serovar E (pSW2). The results from this study suggest that the seroprevalence rate of antibodies in 15-year-olds is similar to the seroprevalence rate in 17-18-year-olds reported in a previous study and therefore the standards of chlamydial screening currently applied to 16-24-year-olds should also be applied to 15-year-olds.

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PUBLICATIONS AND PRESENTATIONS

Some of the work in this thesis has been published:

- Winstanley CE, Ramsey KH, Marsh P, Clarke IN. Development and evaluation of an enzyme-linked immunosorbant assay for the detection of antibodies to a common urogenital derivative of *Chlamydia trachomatis* plasmid-encoded PGP3. J Immunol Methods. 2017; S0022-1759(16)30364-7.

Some of the work in this thesis has been presented:

- "Development of an Improved Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to *Chlamydia trachomatis* Plasmid-Encoded PGP3." – Poster, Faculty Research Conference, University of Southampton, June 2016.

- "Development of an Improved Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to *Chlamydia trachomatis* Plasmid-Encoded PGP3." - Poster, European Society for Chlamydial Research, Oxford, September 2016.

- "Development of an Improved Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to *Chlamydia trachomatis* Plasmid-Encoded PGP3." - Talk, Public Health Application of *Chlamydia trachomatis* Serology Meeting, Oxford, September 2016.

- "Seroprevalence of Antibodies to a Urogenital Derivative of *Chlamydia trachomatis* Plasmid-Encoded PGP3 in 15-year-old Children in England." – Talk, European Society for Chlamydial Research, Oxford, September 2016.

DECLARATION OF AUTHORSHIP

I, CATHERINE WINSTANLEY, declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

The Seroepidemiology of *Chlamydia trachomatis* of 13-15-year-olds in England.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;

2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;

3. Where I have consulted the published work of others, this is always clearly attributed;

4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;

5. I have acknowledged all main sources of help;

6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

7. Parts of this work have been published as: Winstanley *et al*. (2017). J Immunol Methods. 2017; S0022-1759(16)30364-7.

Signed:

Date:

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ABBREVIATIONS

Alkaline phosphatase	**AP**
Ammonium persulphate	**APS**
Analysis of variance	**ANOVA**
Antigen	**Ag**
Base pair(s)	**bp(s)**
β-mercaptoethanol	**BME**
Bicinchoninic acid protein assay	**BCA**
Bovine serum albumin	**BSA**
5-Bromo-4-chloro-3-indolyl phosphate	**BCIP**
Coding sequence	**CDS**
Coefficient of variation	**CV**
Colony forming units	**CFU**
Confidence interval	**CI**
4',6-diamidino-2-phenylindole	**DAPI**
Dimethylformamide	**DMF**
Dimethyl sulphoxide	**DMSO**
Dithiothreitol	**DTT**
Dulbecco's modified Eagle's medium	**DMEM**
Elementary bodies	**EBs**
Enhanced chemiluminescence	**ECL**
Enzyme-linked immunosorbant assay	**ELISA**
Ethics and Research Governance Online	**ERGO**
Ethylenediaminetetraacetic acid	**EDTA**
European Surveillance of Sexually Transmitted Infections	**ESSTI**
Fetal calf serum	**FCS**

Fluorescein isothiocyanate	**FITC**
Genitourinary medicine clinic	**GUM**
Gluathione s-transferase	**GST**
Green fluorescent protein	**GFP**
Health Protection Agency	**HPA**
Health Research Authority	**HRA**
Heat shock protein	**HSP**
Horseradish peroxidase	**HRP**
Human Tissue Authority	**HTA**
Immunoglobulin	**Ig**
Inclusion forming units	**IFU**
Integrated Research Application System	**IRAS**
Interferon	**IFN**
Interleukin	**IL**
Isoelectric point	**pI**
Isopropyl β-D-1-thiogalactopyranoside	**IPTG**
Kilo base pairs	**Kbp**
Kilodalton	**kDa**
Lipopolysaccharide	**LPS**
Lymphogranuloma venereum	**LGV**
Macrophage infectivity potentiator	**MIP**
Major outer membrane protein	**MOMP**
Men who have sex with men	**MSM**
Million base pairs	**Mbp**
Microimmunofluorescence	**MIF**
Monoclonal antibody	**mAb**
Mouse norovirus	**MNV**

Multiple cloning site	**MCS**
National Centre for Biotechnology Information	**NCBI**
National Chlamydia Screening Programme	**NCSP**
National Research Ethics Service	**NRES**
Nitro blue tetrazolium chloride	**NBT**
Non-human primates	**NHP**
Nucleic acid amplification test	**NAAT**
Open reading frame	**ORF**
Optical density	**OD**
Outer membrane protein	**OMP**
Phosphate buffered saline	**PBS**
Plasmid-containing	**P+**
Plasmid-deficient	**P-**
Plasmid growth protein	**Pgp**
Polyclonal antibody	**pAb**
Polymerase Chain Reaction	**PCR**
Polyvinylidene difluoride	**PVDF**
Prescission Protease	**PSP**
Public Health England	**PHE**
Quality control	**QC**
Receiver operating characteristic	**ROC**
Research and development	**R&D**
Reticulate bodies	**RBs**
Room temperature	**RT**
Sodium dodecyl sulfate polyacrylamide gel electrophoresis	**SDS-PAGE**
Standard deviation	**SD**
Standard error of the mean	**SEM**

Sweden	**SW**
3,3’,5,5’-Tetramethylbenzidine	**TMB**
N,N,N',N'-Tetramethylethylenediamine	**TEMED**
Tris base/acetic acid/EDTA	**TAE**
Tumour necrosis factor	**TNF**
Ultra high-quality H_20	**UHQ**
Western blot	**WB**
Women who have sex with men and women	**WSMW**
Women who have sex with women	**WSW**
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG)	**X-Gal**

CHAPTER ONE: INTRODUCTION

1.1 HISTORY OF CHLAMYDIAL RESEARCH

Chlamydiae (‘cloak’) are obligate intracellular Gram negative bacteria which were first observed as inclusions in ocular tissues by Halberstadter and Prowazek in 1907 (1). They named these inclusions Chlamydozoa as they were thought to be protozoans. They also described the transmission of trachoma (‘rough eye’) from humans to orang-utans by inoculating their eyes with conjunctival scrapings. It was later thought that these inclusions were caused by viruses as initial efforts to artificially culture these agents had failed and similar inclusions were later identified in conjunctival epithelial cells of newborns and in the genital tract epithelial cells of their mothers with non-gonococcal urethritis. In 1965, due to advances in tissue culture techniques and of electron microscopy, chlamydia was reclassified as bacteria when they were discovered to contain both DNA and RNA, ribosomes and cell walls (2).

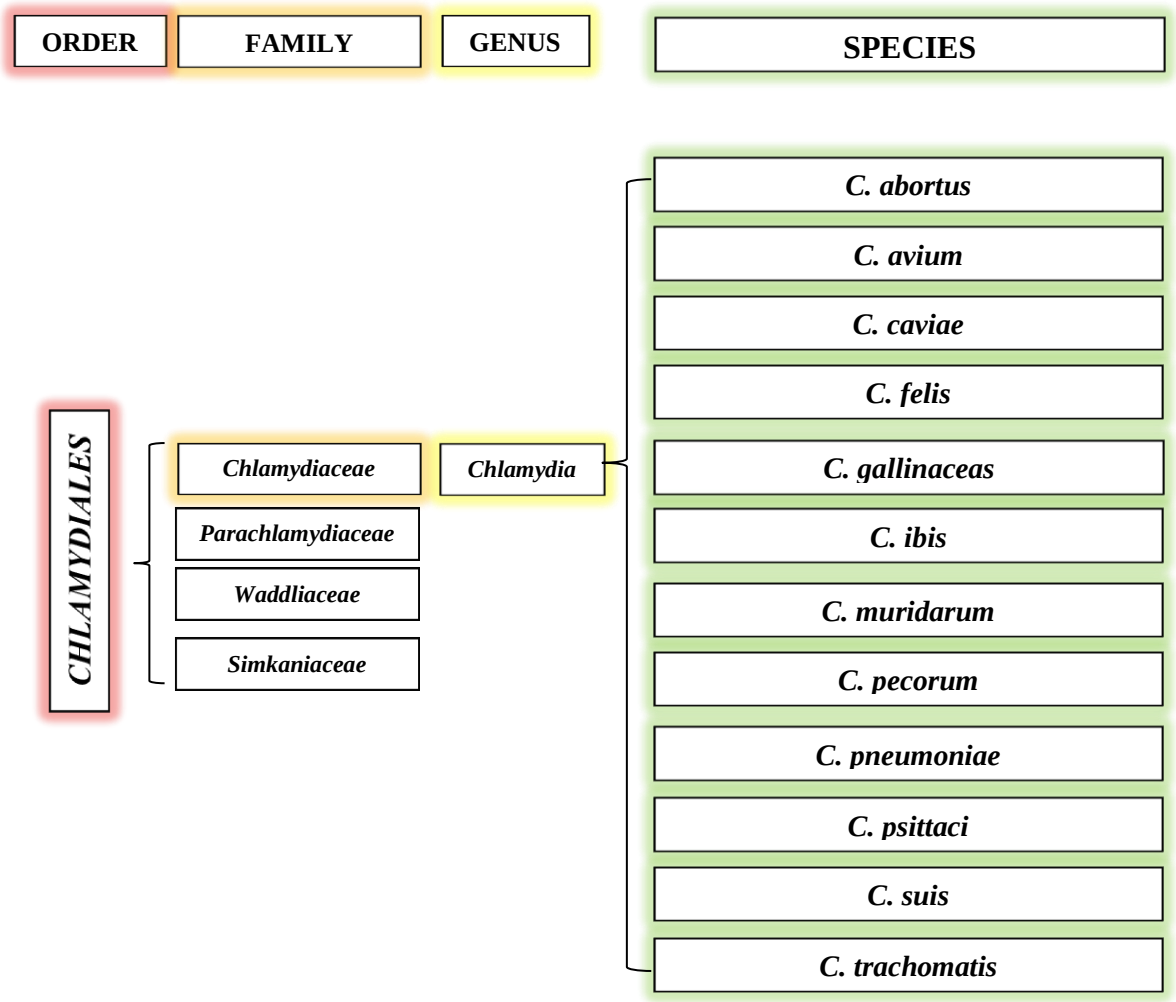
1.2 TAXONOMY

Until the late 1960s, *Chlamydiae* were originally grouped with *Rickettsia* until L.A Page (3) established the order *Chlamydiaeles* consisting of the family *Chlamydiaceae,* and the genus *Chlamydia,* after the discovery of its unique biphasic developmental cycle (4). When the *Chlamydia* genus was established, only two species, *C. trachomatis* and *C. psittaci*, were known. These were distinguishable on the grounds that, unlike *C. psittaci, C. trachomatis* isolates were sensitive to sulphadiazine and formed glycogen-containing inclusions that could be stained with iodine. A third species, *C. pneumoniae*, TWAR (Taiwan acute respiratory agent) was identified in the late 1980s as the causative agent of over 10% of pneumonia cases requiring outpatient treatment or hospitalisation in North America (5). Advances in DNA-DNA hybridisation and 16S rRNA gene sequencing led to the identification of a total of twelve species. These include: *C. suis*, a common agent of intestinal infections in domestic pigs (6); *C. felis*, a common cause of conjunctivitis in cats (7); *C. caviae* which was thought to infect only guinea pigs but has recently been isolated from horses (8); *C. pecorum* which was first isolated from ruminants (9); *C. abortus*, a common cause of spontaneous abortion in cattle and sheep (10); *C. ibis* which was recently isolated from African Sacred Ibises (11); *C. gallinacea* recently isolated from chickens

(12); and *C. avium* isolated from parrots (13). The species *C. muridarum* is the causative agent of pneumonitis and chlamydial genital tract infections in mice and was originally classified as the third biovar of *C. trachomatis*. Since its re-classification, *C. muridarum* has been studied extensively in mice as a model of chlamydial infection. Experiments using mouse models will be mentioned throughout this chapter.

In 1999, Everett et al. (14) made a proposal to divide chlamydial strains in the single genus, *Chlamydia*, into two new genera based on sequence differences between 16S and 23S rRNA sequence genes: the *Chlamydia* genera consisting of *C. trachomatis*, *C. muridarum* and *C. suis*; and the *Chlamydophila* genera consisting of *C. pecorum*, *C. pneumoniae*, and *C. psittaci*, with *C. abortus*, *C. caviae* and *C. felis* deriving from *Chlamydia psittaci*. The proposal was later strongly rejected by many in the scientific community (15) as the new classification proposal was based on minor sequence differences between 16S and 23S rRNA genes. Furthermore, as the proposal included too few isolates to support the authors' statements for reclassification, Schachter *et al.* state that confidence in these minor differences may not be maintained as more isolates are evaluated. Additionally, Schachter *et al.* claimed that the reclassificiation would only create confusion amongst healthcare workers, funders and the scientific community. The classifications currently used are seen in Figure 1.1 (16). Despite this, since the early 2000s a number of studies have been published using the alternative classification of the two genera. In 2015, Sachse *et al.* (17) proposed that the two genera should be unified into a single genus, *Chlamydia*, on the basis that 16s rRNA nor genomic sequencing could provide enough distinction between the two genera. Furthermore, a lack of phenotypic differences between the two genera such as host preference or tissue tropism could support the subdivision.

Figure 1.1. Classification of *Chlamydiales* and current known species of the genus *Chlamydia*. From Stephens *et al.* (16), Sachse *et al*. (17) and Vorimore *et al*. (11).



1.3 MORPHOLOGY

C. trachomatis is pleomorphic and exists in two morphological forms during the developmental cycle: the infectious elementary body (EB) and the non-infectious reticulate body (RB), as seen in Figure 1.2. EBs have historically been described as metabolically inert but a recent study has demonstrated the host-free metabolic activity of EBs (18). There are distinctive features between the two forms (19). The EBs measure 200-300 nm in diameter and contain a thick, granular and rigid cell wall that allows survival in the extracellular environment (20). Hexagonal arrayed rosette structures of omcB and inter- and intramolecular cysteine bonds found in the ompA, omcA and omcB in the outer membrane contribute to the rigidity of the EB (21). Under an electron microscope, the EBs appear spherical and possess hexagonally-organised surface projections that measure approximately 30 nm in length, similar to the type III secretion systems seen in *Salmonella enterica* Typhimurium. RBs measure approximately 1 µm in diameter and are more fragile than EBs with a thinner inner and outer membrane. The cytoplasm of the RBs appear granular under electron microscopy due to ribosome synthesis (19). Like EBs, RBs also possess surface projections that extend from the membrane but at a higher density than the EBs.

1.4 DEVELOPMENT CYCLE OF *C. TRACHOMATIS*

C. trachomatis is an obligate intracellular pathogen and primarily affects ocular and genital tract epithelial tissues in non-human primates. *Chlamydia* share a biphasic developmental cycle which involves several stages, as seen in Figure 1.3. (19).

Infection begins when an infectious elementary body (EB) is taken up by a cytoplasmic vesicle, also known as an inclusion, into a host epithelial cell. Internalisation of *C. trachomatis* allows the cells to evade detection by the host immune responses. The initial primary attachment of the chlamydia to the host cell involves electrostatic interactions between the outer membrane and heparin sulphate-containing glycosaminoglycan receptors on epithelial cells (22, 23). Although the secondary attachment process is not fully understood, it is thought that *C. trachomatis* use a type III secretion system to deliver effectors into the host cell surface that initiate internalisation of *C. trachomatis* using a ‘trigger’ mechanism that involves actin reorganisation inside the host cell (24). After 6-9 hours, the chlamydial DNA decondenses and gene expression initiates as the EB undergoes primary differentiation

and transitions into the non-infectious replicating reticulate body (RB). The RB loses the rigid thick outer membrane that was necessary for survival in the extracellular environment as an EB. The vesicle becomes an expanding inclusion as the RB divides by binary fission. The developmental cycle typically involves 8-10 chromosomal divisions with each division taking approximately 2-3 hours. After approximately 20 hours into the developmental cycle, the chlamydial DNA condenses and the RBs undergo secondary differentiation and reorganise to form 'intermediate bodies' before converting back into infectious EBs. At the end of the cycle, the inclusion may contain up to 500 infectious EBs per host cell and the inclusion occupies up to three quarters of the volume of the host cell. These EBs are released back into the extracellular environment by cell lysis. The whole developmental cycle of *C. trachomatis* occurs over 30-72 hours and can be observed in the time lapse video by Skilton *et al.* (2009). Some stills of this video are included in Figure 1.3. (25).

Figure 1.2. Electron micrograph of an infected HeLa 299 cell. Electron micrographs are modified from (26)**.** Chlamydial classification, development and structure (19). **[A] (i)** Matured elementary bodies are 200-300 nm in diameter and have an electron dense core (black) of nucleic acid condensed onto chlamydial histones. **(ii)** At 2-3 hours post-infection, elementary bodies begin to differentiate into reticulate bodies. The less-dense spotty cytoplasm is due to protein synthesis. **(iii)** The elementary body has enlarged and the cytoplasm is even less dense as chlamydial DNA becomes less prominent. **(m)** Chlamydial cell membrane. The bar represents 0.1 microns. **[B] (R)** The elementary bodies have differentiated into reticulate bodies measuring 1 micron in diameter. **(ds)** Some of the chlamydiae have begun dividing by binary fission. **(e)** Endosomal membrane. **(m)** Vesicles in the host cell membrane. The bar represents 1 micron.

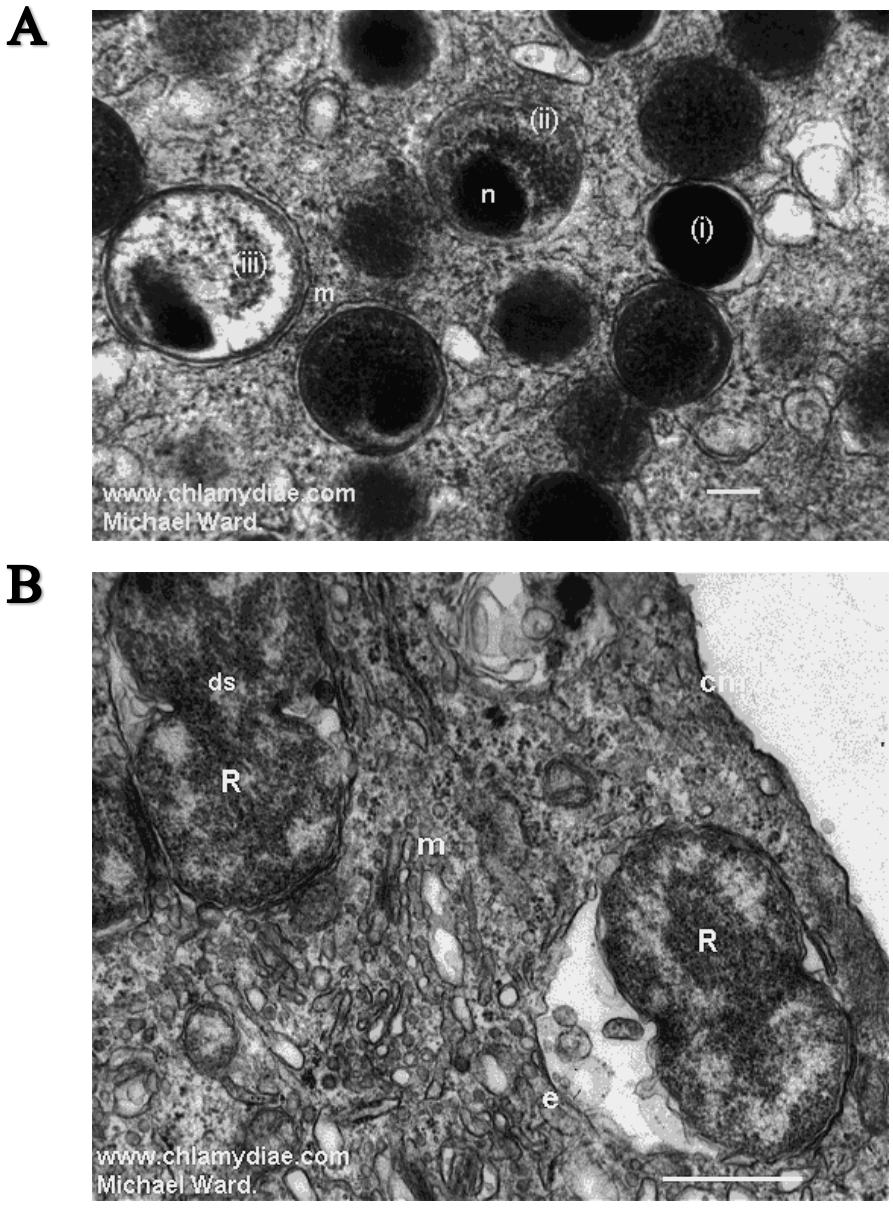
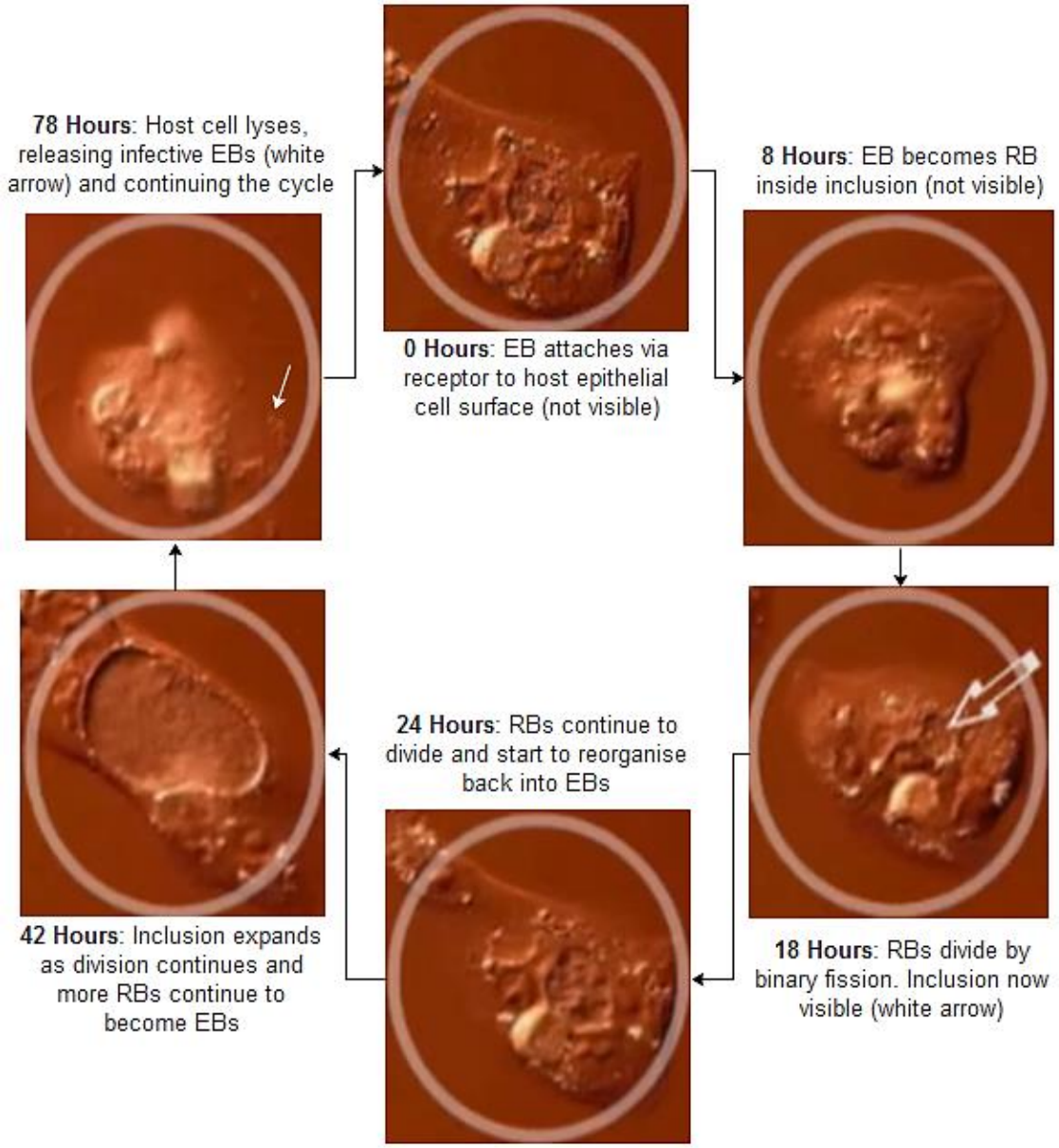


Figure 1.3. The developmental cycle of *C. trachomatis*.

Video stills were included in this figure with permission from Rachel Skilton (25) (http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0007723).



1.5 BIOVARS AND SEROVARS OF *C. TRACHOMATIS*

C. trachomatis isolates are grouped by their biological properties into two biovars: trachoma and lymphogranuloma venereum (LGV). These biovars are further divided into at least 15 serovars. *C. trachomatis* isolates from the trachoma biovar comprise two separate subgroups: serovars A, B and C primarily affecting ocular tissues and serovars D, E, F, G, H, Ia, J and K commonly associated with sexually transmitted urogenital tract infections. The LGV biovar is much less common in the general population and includes serovars L1, L2, L2b and L3 which are able to invade the lymphatic system. The L2b variant has recently been identified as the cause of the outbreak seen in the Netherlands and North America (27). Of the 1 Mbp genome, a total of 4860 single nucleotide polymorphisms (SNPs) have been identified between the trachoma and LGV biovars (28). Serovars have traditionally been classified by microimmunofluorescence (MIF). The diversity of the major outer membrane protein (MOMP) and its gene, *ompA*, has enabled chlamydia to be separated into these serovars using monoclonal anti-MOMP antibodies. MIF has now been replaced with *ompA* sequencing. However, *ompA* sequencing is now considered misleading as it is known that recombination occurs within this gene and it does not provide information about the other 99.85% of the chlamydial genome (28). Furthermore, no correlation between *ompA* differences and the severity of disease has been confirmed (29). However, *C. trachomatis* serovars E, F, D and Ia are the most prevalent genital tract strains worldwide, with serovar E responsible for around one third of cases (30-32). *C. trachomatis* from serovar E has also been found to be predominant in asymptomatic women aged 17-30 whereas *C. trachomatis* from serovar F was predominant in women aged 17-68 attending an outpatient clinic (33). However, as both groups included women from different age groups, there is a possibility these differences were due to urogenital strains of *C. trachomatis* circulating in different age groups of the population and not necessarily tissue tropism.

1.6 PATHOGENESIS OF CHLAMYDIAL INFECTIONS IN HUMANS

1.6.1 Chlamydia pneumoniae

C. pneumoniae was identified in the late 1980s and was linked to over 10% of pneumonia cases requiring outpatient treatment or hospitalisation in North America (5). Chronic disease from *C. pneumoniae* has been linked to adult onset asthma (34, 35) and lung cancer (36). It was previously thought that *C. pneumoniae* was linked to atherosclerosis (37, 38). However, this link was dismissed when a study revealed no significant differences between serum IgG titres to *C. pneumoniae* from over 5600 male patients who died from coronary heart disease or who had non-fatal myocardial infarction and titres from patients without signs of heart disease (39). *C. pneumoniae* has also been isolated from horses (40), frogs (41) and koalas (42). It has recently been suggested that *C. pneumoniae* in humans was acquired zoonotically from animal isolates of *C. pneumoniae* (43).

1.6.2 Chlamydia trachomatis: ocular infections

Trachoma, also known as granular conjunctivitis or blinding trachoma, is caused by serovars A-C of *C. trachomatis* which primarily affect ocular epithelial tissues. Previously and incorrectly classified as being caused by a virus, it is an ancient disease and is known to have been widespread in ancient Rome and Greece and is mentioned in the Egyptian Ebers papyrus dating back to 1550 BC (44). Infection can lead to opacity of the eye, discharge, severe pain, scarring and inflammation of the inner eyelids which can lead to inverted eyelids and eventual blindness (45) . Trachoma is rare in developed countries but it is the most common cause of preventable blindness in the developing world. However, trachoma can be treated with a single dose of azithromycin. Mass treatments in endemic areas have shown some success and trachoma is being targeted for elimination by 2020 (46). This has still proven to be challenging as communities who are undergoing mass drug administration (MDA) are at risk of re-infection from migrant children from other communities where trachoma is endemic (47).

1.6.3 Chlamydia trachomatis: genital tract infections

C. trachomatis serovars D-K commonly associated with sexually transmitted genital tract infections are spread by unprotected sex but are treated effectively with a single dose of azithromycin or doxycycline. Although infections have been reported to be asymptomatic in up to 74% of cases (48), symptoms can include painful urination, abnormal discharge, painful intercourse and abnormal bleeding after intercourse and between periods. However, as asymptomatic cases of chlamydia are often undetected, infection spreads through the population without intervention. In some cases, chlamydial infections are known to self-clear (49, 50) and untreated infections have the potential to spread to the uterus and fallopian tubes leading onto more serious complications such as infertility, pelvic inflammatory disease (PID) and ectopic pregnancy (51-53). Infection can also be transmitted to neonates through childbirth, leading to neonatal conjunctivitis (54, 55).

1.6.4 Chlamydia trachomatis: lymphogranuloma venereum

The lymphogranuloma venereum (LGV) biovar of *C. trachomatis* includes serovars L1, L2 and L3 which are able to infect and invade the lymphatic system. Symptoms can include fever and inflammation of the lymph nodes but it has been reported to be asymptomatic in up to one third of cases (56). It is more common in some parts of the developing world (e.g. Africa, India, South East Asia). However, recent outbreaks of LGV in the Netherlands (57) and North America (58) seen in high-risk populations of men who have sex with men (MSM) were caused by a variant of the L2 serotype, L2b. Cases have also been reported in the United Kingdom and it is thought that many cases go undiagnosed due to routine diagnosis of chlamydia using only urethral swabs rather than urethral and rectal in MSMs (59). Individuals diagnosed with the L2b variant are commonly co-infected with HIV and other sexually transmitted infections, including gonorrhoea, hepatitis C, syphilis and genital herpes (60).

1.7 HOST IMMUNE RESPONSES AGAINST *C. TRACHOMATIS* INFECTION

C. trachomatis has adapted to survive intracellularly inside epithelial cells. As a result, the host immune system incorporates many components of the innate and adaptive immune systems in an effort to eradicate the infection.

Mucosal surfaces of the male and female reproductive tracts serve as the first line of defence against urogenital infection with *C. trachomatis.* Neutrophils and natural killer (NK) cells are the first components of the innate immune response to be recruited to the site of infection (61). Neutrophils are thought to reduce the initial spread of chlamydial infection, as experiments with neutrophil-deficient mice resulted in a chlamydial burden ten times higher than the burdens from non-neutrophil-deficient controls (62). The same study also found that recruitment of neutrophils to the site of infection is dependent on integrin beta-2 (CD18), a surface protein involved in cell-cell adhesion and signalling. However, both experimental groups eradicated the chlamydial infection simultaneously, suggesting an important but non-critical role for neutrophils in clearing infection. Interferon-gamma (IFN-γ) produced by activated neutrophils and NK cells plays several important roles in both innate and adaptive immunity and is characteristic of the Th1 immune response required for the eradication of the infection. IFN-γ has been shown to activate inducible nitric oxide synthase (iNO_3), an enzyme that catalyses the production of nitric oxide, an antimicrobial agent. However, it has recently been suggested that the production of nitric oxide through iNOS may reduce smooth muscle contraction in the fallopian tubes and therefore increase the likelihood of ectopic pregnancy (63). IFN-γ has also been shown to down-regulate the transferrin receptor (64). This may limit the availability of intracellular iron to the chlamydia which is necessary for its survival (65). Similar to neutrophils, mice deficient in IFN-γ resulted in a higher chlamydial burden than the wild-type mice (66). However, only some of the IFN-γ-knockout mice were able to resolve the infection, suggesting an important role for IFN-γ. The release of IFN-γ stimulates the recruitment of macrophages to the site of infection to phagocytose the chlamydia. This releases chlamydial EBs and exposes other antigens including macrophage infectivity potentiator (MIP) to macrophages which have been shown to release cytokines including interleukin (IL)-1β, IL-6, IL-8, and the pro-inflammatory tumour necrosis factor (TNF)-α in response *in vitro* (67). IL-8 is secreted by infected epithelial cells which further recruits NK and dendritic cells (DCs) to the

site of infection in the mucosa. Collectively, the innate immune system works as a positive feedback system to eliminate the chlamydial infection.

IFN-γ has also been shown to affect host epithelial cells *in vitro* by inducing the expression of indole-2,3-dioxygenase (IDO) (68). IDO is an enzyme that catalyses the degradation of tryptophan, an amino acid, into N-formalkynurenine and kynurenine. Tryptophan is essential for the chlamydia to differentiate from reticulate bodies back into infectious elementary bodies (69). Genital tract strains of chlamydia are under selective pressure to retain tryptophan synthase. Interestingly, *trpBA* in genital tract strains encodes functional tryptophan synthase whereas *trpBA* in ocular chlamydial strains contain a number of polymorphisms and results in non-functional tryptophan synthase (70). *In vitro*, the growth of chlamydia is inhibited in tryptophan-deficient medium. However, growth of the genital tract strains of *C. trachomatis*, but not ocular strains, was restored after the addition of indole. Some chlamydial species have adapted to tryptophan depletion by forming dormant cells, sometimes referred to as a persistent infection. These dormant cells are viable but culture-negative and exist as larger reticulate bodies, called aberrant bodies (ABs) (71). ABs are formed during stress, for example antibiotic treatment, amino acid depletion, heat shock or the presence of pro-inflammatory cytokines. During a persistent infection, the ABs lie dormant undetected inside epithelial cells and as the immune response decreases the chlamydia reactivate in a continuous cycle. It is thought that this continuous cycle leads to epithelial tissue damage and eventual scarring (72).

The adaptive immune response comprises of cell-mediated immunity and humoral immunity. The role of adaptive immunity is to limit the spread of infection and provide an enhanced immune response in the case of subsequent reinfections. Chlamydial antigens present on EBs or RBs from lysed epithelial cells released into the extracellular environment as a result of innate immune responses are recognised by the class II major histocompatibility complex (MHC) on antigen-presenting cells (APCs), such as phagocytes and dendritic cells (61). APCs then present the antigen to CD4+ T lymphocytes (T cells) as part of the cell-mediated immune response. CD8+ T cells are activated by chlamydial antigen presentation from within the infected host cell cytosol following recognition by class I MHC. Studies have shown that both CD4+ and CD8+ T cells are present at the site of chlamydial infection. Activated CD4+ T cells proliferate and become effector CD4+ T cells to produce a Th1-dominated immune response by

releasing more IFN-γ. This further recruits more T cells to the site of infection. As intracellular chlamydia are confined to the inclusion, it has been suggested that if CD8+ T cells recognise chlamydial antigens from host cytosolic proteins then it is likely that those proteins have access to the host cell cytosol (73). A potential example of such an antigen could be PGP3, a *C. trachomatis* plasmid-encoded protein that has been reported to be secreted into the host cell cytosol, although the mechanism of secretion is unknown (74). Furthermore, most infected individuals produce antibodies to PGP3 (75). However, the presence of antibodies to chlamydial antigens alone is not sufficient to prevent an infection, although the presence of antibodies has been shown to reduce the spread of infection (76). B cells are able to modulate immunity using three main mechanisms (61): antibody-mediated neutralisation of chlamydial antigens; antibody-dependent cellular cytotoxicity for cell lysis; and aiding antigen recognition by forming antigen-antibody complexes recognised by APCs. Following antigen recognition by T cells, B cells migrate to the lymphoids to undergo somatic hypermutation to generate high-affinity binding sites on antibodies (77). During the early stages of a chlamydial infection, immunoglobulin M (IgM) is produced but these antibodies have a low specificity and deplete after several weeks. IgG and secretory IgA replace IgM after 6-8 weeks and are often targets for antibody detection as an indicator of prior *C. trachomatis* infection using a serological method such as ELISA. Due to the higher abundance of IgG found in serum and the faster seroconversion rate of IgM to IgG, IgG is more commonly used for antibody detection (78).

1.8 VACCINE DEVELOPMENT

Serum IgG and IgA levels have been used to monitor responses to chlamydial candidate vaccines following immunisation. To date, no commercial chlamydial vaccine exists and attempts to produce a vaccine have had limited success. Early chlamydial vaccines in the 1960s focused on inactivated or whole live chlamydial cells to prevent trachoma infection (30). Although one trial using an 'egg-grown trachoma virus vaccine' did show some effectiveness in reducing trachoma in school-aged children in Taiwan (79), children who did develop trachoma had a higher severity of the disease than the unvaccinated controls which may have been induced by vaccine-induced hypersensitivity (80). Furthermore, chlamydial viability has also been shown to play a role in cell-mediated immunity *in vitro* as live EBs were able to cause higher levels of neutrophil and dendritic cell infiltration during infection (81). As technology has since

allowed the isolation and purification of chlamydial antigens, attention has been refocused from whole cells of chlamydia to the development of vaccines using chlamydial antigens or subunits. There are many factors to consider when developing a chlamydial vaccine; the chlamydial antigen, dosage, route of immunisation, number of booster vaccines and the choice of animal model have all resulted in varying reports of protected immunity, as reviewed in (30). Different routes of immunisation in humans have been shown to result in varying levels of IgG and IgA responses (82). Ideally, a chlamydial vaccine should provide protection against the many serotypes of *C. trachomatis*. The major outer membrane protein (MOMP) is antigenically diverse and is found in abundance on the cell surface of all chlamydial serovars. The diversity in the MOMP gene, *ompA*, has allowed the differentiation of chlamydia into serovars. Although significant attention has been given to MOMP as a vaccine candidate (30), reports of immunity provided by MOMP are diverse, suggesting that MOMP alone as a vaccine component would not be sufficient to induce protective immunity. Very recently, attention has been focused back onto using ultraviolet light (UV)-inactivated whole chlamydial cells as a vaccine candidate. Similar to previous experiments in the 1960s, one study has shown that mucosal exposure to UV-inactivated chlamydia exacerbated the subsequent chlamydial challenge. However, exposure to UV-inactivated chlamydia conjugated to charge-switching synthetic adjuvant particles (sSAPs) provided some protection from chlamydial challenge in mice (83). It is thought that this newly developed technology enhances the phagocytic ability of dendritic cells. Attention has also been given to the chlamydial plasmid-encoded protein, PGP3, as a vaccine candidate. Immunisation with PGP3 has shown reduced bacterial burdens in the lungs of infected mice, although protection was found to be due to CD4+ T cell-mediated immunity and not due to the presence of anti-PGP3 antibodies (84). Although chlamydial infection was not prevented, DNA immunisation incorporating the PGP3 gene provided partial protection against chlamydial challenge in mice in two studies (85, 86).

1.9 EPIDEMIOLOGY OF UROGENITAL *C. TRACHOMATIS* INFECTIONS

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 (87, 88). *C. trachomatis* infections are often asymptomatic in many instances and such cases often go undiagnosed, enabling the infection to spread through the population without treatment (48). Untreated infections have the potential to lead to more serious complications such as infertility, pelvic inflammatory disease and ectopic pregnancy (51). Collectively, the treatment and management of these preventable chlamydial infections and their associated comorbidities place a significant burden on healthcare systems.

1.9.1 Heterosexual population

In 2012-2014, each year there were over 200,000 diagnosed cases of genital chlamydial infection in England alone, as seen in Table 1.1 (89). There has been a dramatic increase in chlamydial testing, rising from an estimated 1.8% of all 15 to 24-year-olds in 2005 to 29.9% of all 15 to 24-year-olds in 2010 (90). However, diagnosed rates of chlamydia in the UK have seen a steady increase since 1998, rising from an estimated 100 cases of chlamydia per 100,000 individuals, to 200 cases per 100,000 in 2007 (91). This steady increase has occurred even after the introduction of the National Chlamydia Screening Programme (NCSP) in 2003, although it is important to consider that this steady increase may be a result of increased detection rates from screening and not increased prevalence. Between 2000-2011, a total of over 1.4 million diagnoses of chlamydia were made in England (92). Increased rates of disease burden were found to be associated with socioeconomic deprivation, particularly in large cities such as Manchester and London (89, 93). Comparable to the UK, there have been increased rates of chlamydia across many European countries. In 2008, the European Surveillance of Sexually Transmitted Infections (ESSTI) reported chlamydia to be the most commonly diagnosed STI with 68% of cases reported in the under-25s (91). They also reported a steady increase in chlamydia rates in Ireland, Finland, Sweden, Denmark, Norway and Iceland, whereas rates of chlamydia in Latvia and Slovenia appear to have remained low and stationary, at only 10 cases per 100,000 individuals (Figure 1.4). However, the ESSTI also state that comparisons of rates between countries may be misleading due to differences in surveillance systems, screening and detection rates.

Interestingly, there is a notably sharp increase in reported cases of chlamydial infection in Sweden from 2006 to 2007. In 2006, a new Swedish variant of *C. trachomatis* (nvCT) was identified that was undetectable to PCR diagnostic systems (94). This nvCT was estimated to have spread amongst the population since 2003 undetected and unchallenged (95) which possibly explains the plateau of chlamydial diagnoses rates seen in Sweden from 2004 to 2006. A more detailed discussion of the nvCT can be seen in 1.12.1.

Figure 1.4. Reported cases of *Chlamydia trachomatis* per 100,000 population across Europe, 1998-2007. From the European Surveillance of Sexually Transmitted Infections (ESSTI) (91). Note the dramatic increase in chlamydial diagnoses seen in Sweden between 2006-2007 due to the emergence of the Swedish variant (nvCT).

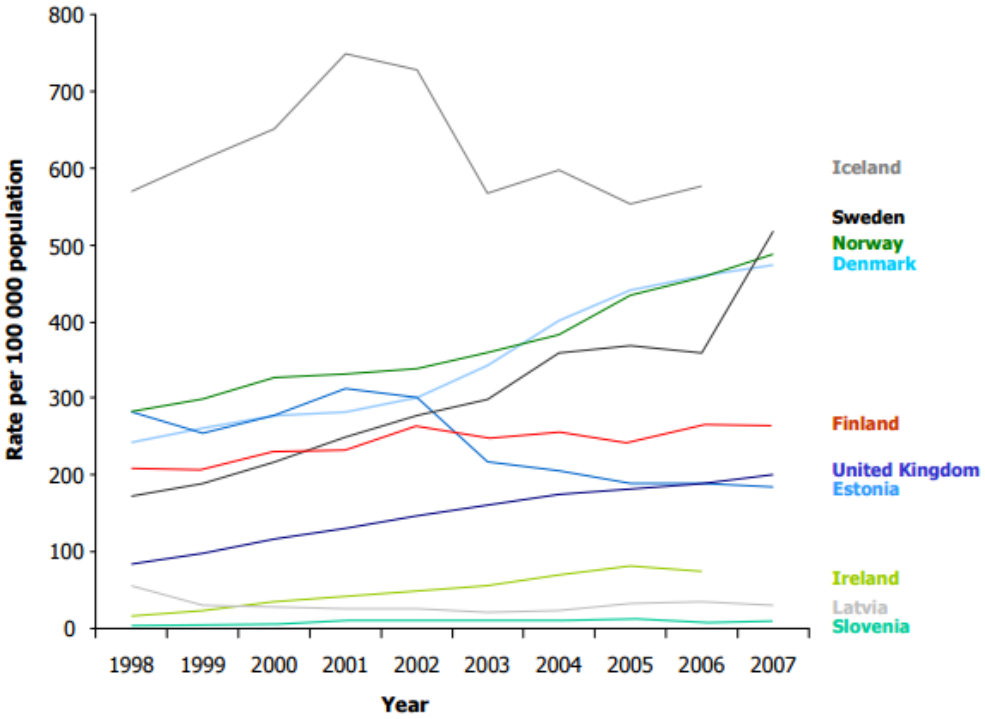


Table 1.1 Total chlamydial diagnoses in England by gender and age group, 2010-2015, Public Health England. (89).* = includes unknown age and 0-12-year-olds.

Age Group	2012		2013		2014		2015	
	Male	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**Female**
13-14	92	912	102	966	56	817	51	616
15-19	15,937	44,445	16,014	44,041	14,724	41,995	13,675	38,311
20-24	34,925	46,831	34,033	47,482	32,921	47,768	31,122	45,235
25-34	24,542	21,293	24,839	22,251	25,869	23,299	26,643	24,002
35-44	6,723	4,254	6,645	4,205	7,279	4,322	7,789	4,494
45-64	3,388	1,553	3,405	1,516	3,806	1,576	4,119	1,544
65+	208	49	238	56	253	45	242	44
Other*	171	211	158	202	198	186	153	263
Total	**85,986**	**119,548**	**85,434**	**120,719**	**85,106**	**120,008**	**83,794**	**114,509**

1.9.2 Men who have sex with men

Studies of the prevalence of *C. trachomatis* in the under-25 age group have understandably been given a great deal of attention. However, it is important not to overlook other age groups not covered by the NCSP and other minority populations who may be at risk, since differences in prevalence have been reported amongst patient backgrounds, including ethnicity, location and sexual preference, where in particular a dramatic increase in diagnosed infections have been reported in the 'men who have sex with men' (MSM) population (89). This dramatic increase has been attributed to the increase in the use of so-called 'chemsex' drugs, sex parties and the use of web and mobile social media applications (96). Although the LGV biovar of *C. trachomatis* is much less common in the general population in the developed world, LGV is becoming more common in populations of men who have sex with men (MSM) (97). The L2b variant is also responsible for the recent outbreak seen in Europe (57) and North America (58) in MSMs and individuals diagnosed with the L2b variant are commonly co-infected with other sexually transmitted infections (60).

1.9.3 Children under 16-years-old

In 2013, over 21,000 young people under 15 were screened for chlamydia infection and nearly 1,000 (4.76%) of these tests were diagnosed as positive, representing 0.2% of the overall population for young people under 15 (89). As the NCSP actively targets people over 15-years-old for screening, this diagnoses are likely to be an underrepresentation of chlamydial infection in the under 15s. Furthermore, 15-year-olds are only screened for chlamydial under certain circumstances and patients may be hesitant to attend appointments as parental notification is often recommended. Research has suggested that the numbers of people having their first sexual encounters before age 16 are increasing. One study found nearly 30% of the 16 to 24-year-old men and women they surveyed experienced their first sexual encounter before age 16 in comparison to only 4% of women currently aged 65 to 74-years-old (98). One of the few studies in England that investigated the rate of chlamydia in under 16s found that amongst the 264 children tested, 20% tested positive for sexually transmitted infections overall and 10% tested positive for *C. trachomatis* infection (99). Of those who tested positive for chlamydia, it was found that 20% of the children had either past or ongoing sexual abuse. Another study in the US assessed urine and genital swabs of 485 female children by NAAT and found a 2.7% prevalence for *C. trachomatis* and 3.3% prevalence of *Neisseria gonorrhoeae* (100). However, all the children tested were suspected to have been sexually abused and so the prevalence data in the study is not likely indication of general prevalence in the under 16s population. As genital chlamydial infections can often self-resolve and especially in cases of current or past sexual abuse, it is possible that the actual numbers of genital chlamydial infection in children under 16 are likely to be higher than the numbers reported by PHE. In this case, serology tests for chlamydial antibodies would be particularly useful to provide a more accurate estimate of incidence of chlamydial infection in children.

1.9.4 Women who have sex with women

In the UK, very little information is available on prevalence of chlamydia infection in the women who have sex with women (WSW) population and no serological studies have yet been conducted. Very few studies have also been conducted in the women who have sex with men and women population (WSMW). This could be due to the social myths and misconceptions that WSWs are less likely to transmit sexually transmitted diseases and are less likely to be as sexually active as ‘women who have sex with men’

(WSM). However, recent surveys and studies could challenge these perceptions. In 2008, a survey by Stonewall of over 6000 WSWs (81%) and WSMW (16%) found that 53% had never been tested for an STI and 15% of those aged 25-64 had never been screened for cervical cancer (101). Despite only 16% of those surveyed identifying as WSMW, nearly 30% stated to having both male and female sexual partners within the last 5 years, and 2% admitted to being exclusively with male sexual partners. If chlamydial infections in WSMW are undiagnosed, it could be possible that the WSMW and WSW with male sexual partners could act as a 'bridge' population between groups where chlamydia is most prevalent (WSM aged 15-24) and groups were chlamydial infections are possibly least prevalent (WSW). In 2004, 708 WSWs and WSMWs attending two sexual health clinics in London were assessed for sexually transmitted infections and no statistical significance was observed in the differences between prevalence of STIs between women who had reported a history with male sexual partners and those who had reported none (102). Of the 708 patients assessed, 4 (0.6%) were found to be positive for chlamydial infection. Despite this seemingly low incidence rate, other studies using information on patient history from public health databases or clinical specimens obtained from recruitment found incidence of chlamydia amongst WSW to be between 3% and 7% (103-105). Since chlamydial infections can self-clear and 53% of WSW and WSMW surveyed had never been screened for an STI (101) the actual incidence rate of chlamydial infection amongst WSW and WSMW may be higher. Again, similar to the under-15s population, serological testing for chlamydial antibodies would be very beneficial to build a better understanding of chlamydial incidence amongst these overlooked populations.

1.9.5 National Chlamydia Screening Programme

With diagnosed rates of chlamydia increasing over the last two decades (91), the National Chlamydia Screening Programme was launched in England by the Department of Health in 2002 to provide free and confidential chlamydial screening for sexually active young people aged 15-24 (106). In its first year, over 16,000 diagnostic tests were performed, of which 93% of tests were from females and an overall 10.43% of tests were positive for chlamydia (107). However, the NCSP has been criticised for their opportunistic testing approach rather than systematic testing, also known as register-based testing, in which patients are recruited for routine annual testing (108). Although this was reviewed, the NCSP chose to remain with the opportunistic approach whilst

recommending annual screening to sexually active under-25s. Furthermore, the under 16s are only tested in accordance with the Fraser Guidelines (109). The Fraser guidelines provide guidance to health professionals in relation to providing and advising contraceptives to the under 16s and, as of 2006, treating sexual health issues. These guidelines, currently not subjected to the 16-24-year-old age group, include confirming that: the patient has the maturity and intelligence to understand the implications of the proposed treatment, the patient cannot be persuaded to tell her parents, the patient is very likely to begin or continue having sexual intercourse with or without contraceptive treatment, the patient's physical or mental health is likely to suffer unless they receive the advice or treatment, and the advice or treatment is in the young person's best interests.

1.10 DIAGNOSTIC AND SEROLOGICAL METHODS

1.10.1 Isolation by culture

C. trachomatis, historically known as the 'trachoma virus', was first isolated in chick-embryonated yolk sacs in 1957 (110). During the 1960s and 1970s, techniques were developed to culture chlamydial organisms using cycloheximide-treated McCoy cells (111). A confluent monolayer of McCoy cells are inoculated with a chlamydial specimen and the culture is centrifuged and cultured at 37°C with 5% CO_2 for up to 72 hours. The inclusions are then detected using a fluorescently-labelled monoclonal antibody specific to the *C. trachomatis* major outer membrane protein (MOMP). Although chlamydial isolation using culture allows viewing of the chlamydia, it is a time-consuming procedure, expensive and requires specially-trained laboratory staff. Furthermore, the chlamydial cells may lose viability during specimen transport and cell culture techniques may vary lab-to-lab, reducing the specificity and reliability of the technique for diagnostic purposes.

1.10.2 Microimmunofluorescence

The microimmunofluorescence (MIF) assay is an indirect assay first developed in the 1970s as a method for the seroepidemiological analysis of *C. trachomatis* infections, although it can be used for other chlamydial species (112). Purified whole chlamydial elementary bodies grown using cell culture techniques are often used as the capture antigens. Patient sera are then applied and bound antibodies are detected by a fluorescently-labelled secondary antibody. However, MIF using patient sera for

diagnosis is neither sensitive nor specific, as the technique relies on the presence of antibodies in patient sera. Furthermore, as whole *C. trachomatis* elementary bodies are used as the capture antigens, there is also the potential of false-positive cross-reactivity to *C. pneumoniae*. The advantages of MIF is that the chlamydia can be stored for years at 4°C and it can detect low levels of IgM antibody, such as in cases of suspected pneumonitis in infants (113, 114). The MIF assay technique is also time-consuming, expensive and methods may vary between laboratories. As discussed earlier, MIF was also used to classify chlamydia into serovars using specific monoclonal antibodies to MOMP, though this has now been replaced with *ompA* sequencing. Direct fluorescent antibody (DFA) detection is similar to MIF in that a fluorescently-labelled detection antibody is used: specimens are spread onto microscope slides and chlamydial inclusions are detected with a monoclonal fluorescently-labelled antibody.

1.10.3 Nucleic acid amplification testing

Nucleic acid amplification testing (NAATs) are a common routine diagnostic method to detect *C. trachomatis* in urine samples or anal, vaginal or urethral swabs (115). NAATs amplify sequences that are specific to the test organism, of which currently include *C. trachomatis* and *Neisseria gonorrhoeae*. Common test systems include the Roche Amplicor (Roche Diagnostics) which uses PCR, the Abbot LCx (Abbott Laboratories) which uses ligase chain reaction (LCR), and the Becton Dickinson BDProbeTec ET (Becton, Dickinson and Company) which uses strand displacement amplification. Targets for amplification include sequences found on the *C. trachomatis* plasmid. As the plasmid is never found in human isolates of *C. pneumoniae* (116), this eliminates concerns of cross-reactivity or false-positive reactions to *C. pneumoniae*. NAATs also only require a single copy of the target DNA, making NAATs a more sensitive and specific diagnostic method than MIF, culture or serological methods.

1.10.4 Nucleic acid hybridisation (NAH) tests

Nucleic acid hybridisation (NAH) tests are similar to NAATs in that the technique detects a specific nucleic acid sequence. In this case, the sequence is chlamydial rRNA. The chemiluminescent-labelled DNA probe binds to the complementary rRNA to form a stable DNA-RNA hybrid. The main difference between NAH and NAATs is that nucleic acid sequences are not amplified in NAH. Disadvantages of the NAH tests are

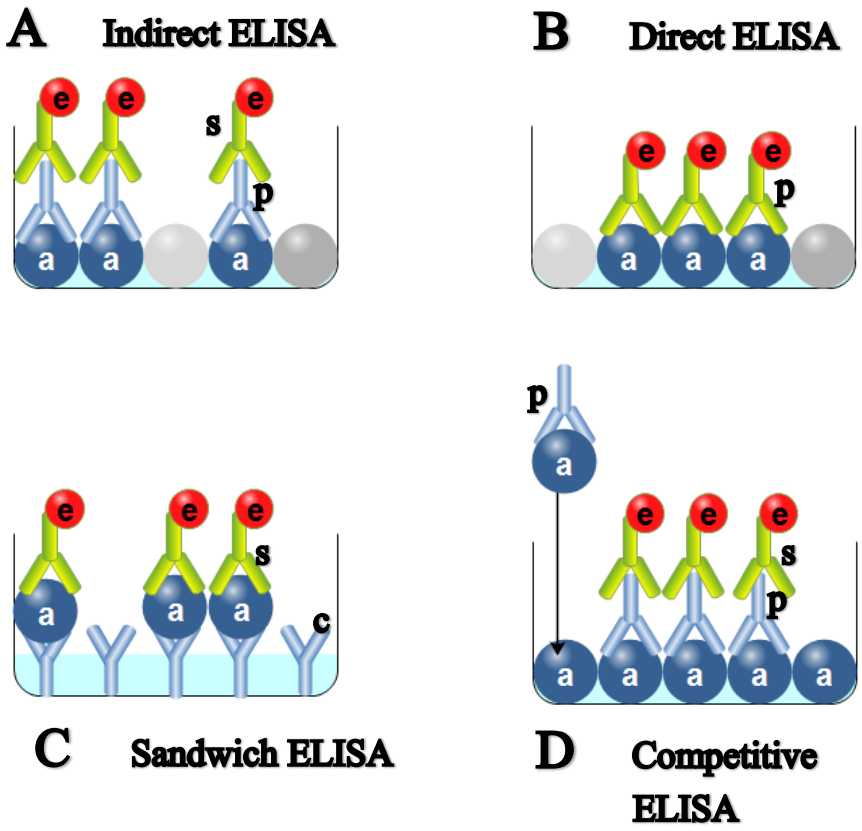
that it requires a high technical ability and the tests are not as sensitive as NAATs (115), although both tests enable samples to be stored for up to one week.

1.10.5 Enzyme-linked immunosorbant assays

Several commercial ELISA kits are available to detect the presence of anti-chlamydial antibodies in patient sera. Similar to MIF, some of these tests use whole chlamydial elementary bodies or components such as MOMP or LPS as the capture antigens. Patient sera are then applied and antibodies are detected using a labelled secondary antibody. As a result, these tests are reliant on the presence of antibodies in patient sera. These tests are also not specific as there is the potential of false-positive reactivity to other chlamydial species, such as *C. pneumoniae* and *C. psittaci* (117). Furthermore, the detection of antibodies to chlamydia alone does not distinguish between a current and previous infection. Commercial kits include the Medac pELISA plus, the Savyon SeroCT-IgG ELISA and the Ani Labsystems IgG enzyme immunoassay, all of which are based on the *C. trachomatis* MOMP and vary in sensitivity and specificity (118). There are several types of ELISAs that are used to detect the presence of antibodies or antigens in samples (Figure 1.5): Indirect ELISA, direct ELISA, competitive ELISA and sandwich ELISA. Indirect ELISAs use one or more capture antigens that detect the presence of a specific or group of antibodies in a sample. Bound antibodies are then detected by a labelled secondary antibody. Direct ELISAs also use a capture antigen but the antigen is detected by a primary antibody that is already labelled. This assay is usually for the detection of a specific antigen within a sample. Sandwich ELISAs utilise a coating antibody to ‘capture’ and detect a specific antigen within an impure sample which is then detected by a labelled second antibody to the antigen. Competitive ELISAs are similar to indirect ELISAs but involve incubating the primary antibody and antigen together before being added to the assay plates.

Figure 1.5. Common types of enzyme-linked immunosorbent assay (ELISA).

[A] Indirect ELISA; **[B]** Direct ELISA; **[C]** Double-Sandwich ELISA; **[D]** Competitive ELISA. All labelled antibodies are conjugated to an enzyme which turns a colourless substrate into a coloured product. Depending on the type of ELISA used, the colour change is proportional to the amount of target antibody or antigen present; the more concentrated the antigen or antibody, the darker the substrate colour. The colour is then read by a spectrophotometer. [a = antigen; p = primary antibody; s = secondary antibody; e = enzyme; c = capture antibody].



1.11 *C. TRACHOMATIS* ANTIGENS AS MARKERS IN SEROEPIDEMIOLOGY

Chlamydial protein markers that are able to produce an immune response in infected individuals play an important role in serology as the presence of antibodies to a particular marker can indicate a current or previous chlamydial infection. However, they are not valuable as a diagnostic tool as they provide no indication of a current infection. As discussed in 1.10.5, many commercial ELISAs incorporate chlamydial antigens as markers but these vary in the antigens used, the assay format and the resulting assay sensitivity and specificity. An ideal serological marker should be highly immunogenic and easy to obtain or purify. Most importantly, it should also be specific to the test species. Several chlamydial antigens have been investigated as candidate antigens for a vaccine (1.8).

As discussed, the major outer membrane protein (MOMP) has received significant attention as a marker of chlamydia infection and is used in several commercial ELISA kits. MOMP produces a robust immune response in infected individuals and it has recently been found to induce IFN-γ and T cell responses in mice (119). Immune responses to MOMP may therefore increase the risk of ectopic pregnancy, as IFN-γ induces the production of NO_3 which has been suggested to reduce smooth muscle contraction in fallopian tubes, as discussed in 1.7 (63). Heat shock protein (hsp) 60 has been suggested as a marker of ectopic pregnancy. One study found that antibodies to hsp60 were more prevalent in women who have experienced an ectopic pregnancy in comparison to women with normal pregnancies (120). All serum samples from both groups also contained antibodies to MOMP. However, the specificities of *C. trachomatis* MOMP and hsp60 as serological markers is in doubt. Baud *et al.* (121) reported good sensitivities and specificities of five commercial tests incorporating hsp60 and MOMP. However, some cross-reactivity to *C. pneumoniae* antibodies was reported and all tests cross-reacted with *C. psittaci* antibodies. In 2001, Bas *et al.* (122, 123) investigated the outer membrane protein 2 (OMP2), hsp10, hsp60, hsp70, macrophage infectivity potentiator (MIP), species-specific fragments of lipopolysaccharide (LPS), species-specific epitopes of MOMP and the plasmid-encoded protein PGP3 as potential serological markers of chlamydial infection. They found that MOMP combined with PGP3 resulted in a sensitivity of 79% and a specificity of 82%. A higher sensitivity of 89% for OMP2 was reported but specificity was lower at 57% due to cross-reactivity with the *C. pneumoniae* OMP2. A high sensitivity of 84% was

also reported for LPS but the specificity was lower at 52%. MIP was only able to detect antibodies in 20% of infected individuals. Bas *et al.* used 'healthy control serum' as their negative control serum cohort. This may explain their reduced specificity as the absence of chlamydial symptoms or a negative NAAT result would not exclude the possibility of anti-chlamydial antibodies. It is important to consider the antibody detection format and the method of obtaining or purifying the capture antigen; Bas *et al.* purified hsp10, hsp60, hsp70, MIP and PGP3 expressed as recombinant fusion proteins with polyhistidine tags. It is not possible to know if these recombinant fusion proteins retained their native structure in an ELISA or immunoblot or whether the fusion polyhistidine tag would alter their native structure. Although a sensitivity of 57% and specificity of 89% were reported for the PGP3 assay, it has been suggested that human antibody recognition of PGP3 is dependent in its native trimeric conformation (124, 125). PGP3 has received significant attention as a serological marker of chlamydial infection, as discussed in further detail in 1.13.3.

1.12 THE CHLAMYDIAL PLASMID

Nearly all *C. trachomatis* strains carry a 7.5 kb plasmid containing eight coding sequences (CDS1-8) (126) which contribute to chlamydial infectivity and regulation of chromosome- and plasmid-encoded genes (127, 128). Alignment of 11 plasmids from 12 serovars of *C. trachomatis* has revealed a total of 83 SNPs, representing an approximate 1.1% variation between the plasmids (126). At least nine species of *Chlamydiaceae* are known to carry the plasmid (129). The *C. muridarum* plasmid, pMoPn, is often used in mouse models of chlamydial infection and shares 80% nucleic acid identity to the *C. trachomatis* plasmid (129). Although the chlamydial plasmid is not essential for growth or survival (130), the importance of the chlamydial plasmid in infectivity and virulence has been demonstrated in various studies, suggesting an important but non-critical role for the plasmid.

The *C. trachomatis* plasmid is present at up to eight copies per genome which is dependent on the stage of the chlamydial development cycle and the chlamydial strain (126, 131), although no associations between plasmid copy number and chlamydial load, disease severity or tissue tropism have been identified (132, 133). However, experiments using mice infected with plasmid-free strains of *C. trachomatis* and *C. muridarum* revealed significantly reduced bacterial burdens in comparison to the

plasmid-containing strains (128, 134-136). Reduced transcription of chromosomal genes has also been observed in plasmid-free *C. trachomatis* strains. Carlson *et al*. (2008) (128) reported reduced transcription of the glycogen synthase gene, *glgA*, in a naturally-occurring plasmid-free *C. trachomatis* isolate (L2 25667R). Consistent with their findings, this isolate was unable to accumulate glycogen within the chlamydial inclusion. Failure to accumulate glycogen inside the chlamydial inclusion has also been observed in *C. trachomatis* SWFP- (137). Interestingly, the morphology of this *C. trachomatis* SWFP- strain is altered in comparison to the plasmid-containing strain, as 'doughnut-shaped' inclusions were reported. The role of the plasmid in host cell lysis at the end of the developmental cycle has also recently been demonstrated (138). Yang *et al.* (2015) reported that plasmid-free *C. trachomatis* were unable to disrupt the host cell cytoskeleton to induce cell lysis. However, lysis occurred when host cell actin polymerisation was inhibited.

The complete functions of all the expressed coding sequences of the chlamydial plasmid are still unknown. However, the recent development of a *C. trachomatis* transformation system based on calcium chloride treatment of EBs (139) has allowed investigations into the functions of these 'plasmid growth proteins' (PGP), as summarised in Table 1.2. CDS5, previously known as ORF3, encodes PGP3 and is discussed in detail in 1.13. PGP3 is the only plasmid-encoded protein secreted outside the chlamydial inclusion and into the host cell cytosol (74), although neither the mechanism of protein secretion nor its primary function is known. The role of PGP3 has remained elusive for many decades, although it has recently been reported that PGP3 binds to and neutralises LL-37, an antimicrobial peptide secreted by epithelial cells and leukocytes (140). CDS6 encodes PGP4, a transcriptional regulator of chromosomal encoded genes which is also required for the expression of PGP3 and accumulation of glycogen within inclusions (137, 141). PGP4 has also recently been found to play a role in host cell lysis at the end of the developmental cycle (138). Very recently, PGP5 has been found to play a role in inducing hydrosalphinx in mice, as mice infected with PGP5-deficient *C. muridarum* revealed significantly reduced levels of ascending chlamydial infection (142). CDS2 is thought to play a role in plasmid species-specificity (143). Although a transformation system for *C. trachomatis* has been developed (139), *C. muridarum* is unable to be transformed with *C. trachomatis* plasmids. Wang *et al.* (2014) investigated regions of the plasmid that may be involved in species-specificity by forcing recombination

between the *C. trachomatis* pSW2 and *C. muridarum* pNigg plasmids and found that a 1 kb region in CDS2 had been exchanged. The chlamydial plasmid also contains four 22 bp tandem repeats which is thought to be the origin of replication between CDS8 and CDS1 (144).

1.12.1 The Swedish Variant

As mentioned in 1.9, a new Swedish variant of *C. trachomatis* (nvCT) was identified that was undetectable to PCR Abbott m2000 (Abbott) and Amplicor/COBAS Amplicor/TaqMan48 (Roche) diagnostic systems (94). Until its discovery in 2006, this nvCT spread undetected and unchallenged amongst the population since 2003 (95). This may explain the plateau of chlamydial diagnoses rates seen in Sweden from 2004 to 2006, followed by the sudden sharp increase in diagnoses in 2007 (Figure 1.4). Up to an estimated 65% of chlamydial cases around this time were caused by the nvCT (145), although this has since fallen to 6% in 2011 (146). However, since 2006 cases of the nvCT have been reported in Germany, Spain and Russia, although at much lower rates (147-149). The spread of the nvCT was due to a 377 bp deletion in the CDS1 region which eliminated one of the primer binding sites used for PCR detection (126). This deletion caused a frameshift in the polypeptide sequence, reducing the size of the expressed protein from 305 amino acids to 178. A 44 bp duplication immediately upstream of CDS3 was also identified in the nvCT. No biological fitness or phenotypical differences were identified in the nvCT when comparing with the *C. trachomatis* serovar E wild-type (94). Furthermore, Sigar *et al*. (2013) (150) reported no differences in chlamydial shedding in mice infected with the nvCT, although slight differences in seroconversion rates were observed. This evidence suggests that the rapid transmission of the nvCT was due to the selective advantage from false-negative diagnosis due to the deletion in CDS1 (151). This would have also been exacerbated by the introduction of the nvCT into a high frequency-transmitting population.

Table 1.2. Summary of the main functions and features of the coding sequences and proteins of the *C. trachomatis* plasmid.

PGP	CDS	Function and other notes	Ref
PGP1	CDS3	Required for plasmid maintenance. Encodes a DnaB-like helicase. 44bp duplication in nvCT.	(141, 152)
PGP2	CDS4	Required for plasmid maintenance.	(141, 143, 152)
PGP3	CDS5	Previously known as ORF3. Produces a 28 kDa protein that presents as a trimer. Only Pgp secreted outside the chlamydial inclusion. Induces antibodies and pro-inflammatory cytokines. Not required for growth. Immunogenic; vaccine candidate. Neutralises LL-37 in the host.	(74, 75, 140, 153, 154)
PGP4	CDS6	Deletion results in inclusion phenotype identical to plasmid-free strains. Transcriptional regulator of *glgA*, required for glycogen synthase. Also regulates PGP3 expression. Immunogenic; vaccine candidate.	(137, 138, 141, 152, 155)
PGP5	CDS7	Induces hydrosalphinx in mice. Negatively regulates some chromosomal encoded genes. Not required for growth.	(142)
PGP6	CDS8	Required for plasmid maintenance.	(141)
PGP7	CDS1	Encodes recombinase. Not required for growth. *C. trachomatis* SW strains (nvCT) contain a 377 bp deletion.	(133, 141, 151)
PGP8	CDS2	Required for plasmid maintenance. Responsible for species-specificity of plasmids.	(133, 141, 156)
N/A	CDS8-1	Origin of replication; contains four 22 bp tandem repeats.	(144)

1.13 PLASMID GROWTH PROTEIN 3 (PGP3) (CDS5)

Plasmid growth protein 3 (PGP3) is encoded by the 795 bp coding sequence 5 (CDS5) of the *C. trachomatis* plasmid. Although the plasmid is conserved amongst *C. trachomatis* strains (126), CDS5 contains the most SNPs across all serovars with a total of less than 5% amino acid variation across all sequences, as seen in figure 1.6, Table 1.3, Table 1.4 and appendix I. 100% sequence similarities are observed between PGP3 derived from serovars A-C, urogenital tract serovars D-F, and LGV serovars L1-L3, but differences are observed between these individual groups. For example, an amino acid sequence similarity of 96.2% is observed between PGP3 derived from pCTA (serovar A) and pLGV440 (serovar L1). 100% sequence similarities are also observed between PGP3 derived from serovars H-I and serovars G, K and J.

The expression of PGP3 is regulated by PGP4, although PGP3 is not required for the transcriptional functions of PGP4 (141). In 1993, Comanducci *et al.* (157) first reported the presence of PGP3, or 'ORF3', within chlamydia after antibodies raised to PGP3 recognised a 28 kDa protein within chlamydial EBs using Western blot assay. The expression of PGP3 was detected at 20 hours post-infection, although another study reported the presence of PGP3 as early as 12 hours post-infection *in vitro* (74). Li *et al.* (74) also reported that PGP3 was secreted into the host cell cytosol by *C. trachomatis*, *C. muridarum* and *C. psittaci* plasmid-containing strains, suggesting a selective pressure for maintaining PGP3 secretion during chlamydial infection.

Figure. 1.6 Phenogram analysis of PGP3 (CDS5) amino acid sequences between serovars A-K and L1-L3 serovars of *C. trachomatis.* Phenogram was constructed using the MegAlign clustering program from the DNAStar Lasergene version 14.0 software package. [pCTA: EMBL CP00052; pJALI: EMBL FM865438; TW-3: Genbank CP006946.1; pCHL1: NC_001372; pSW2: FM865439; pSW4: FM865441; pSOTONG1: H603235; pH: N/A; pSOTONH1: HE603236; pJ: N/A; pSOTONK1: HE603238; pLGV440: EMBL X07547; pL2: EMBL X07547; pUCH-1: EMBL AM886279; pL3404: HE603228. Sequences for pH and pJ plasmids were provided by James Hadfield at the Sanger Institute and are not publicly available online.]

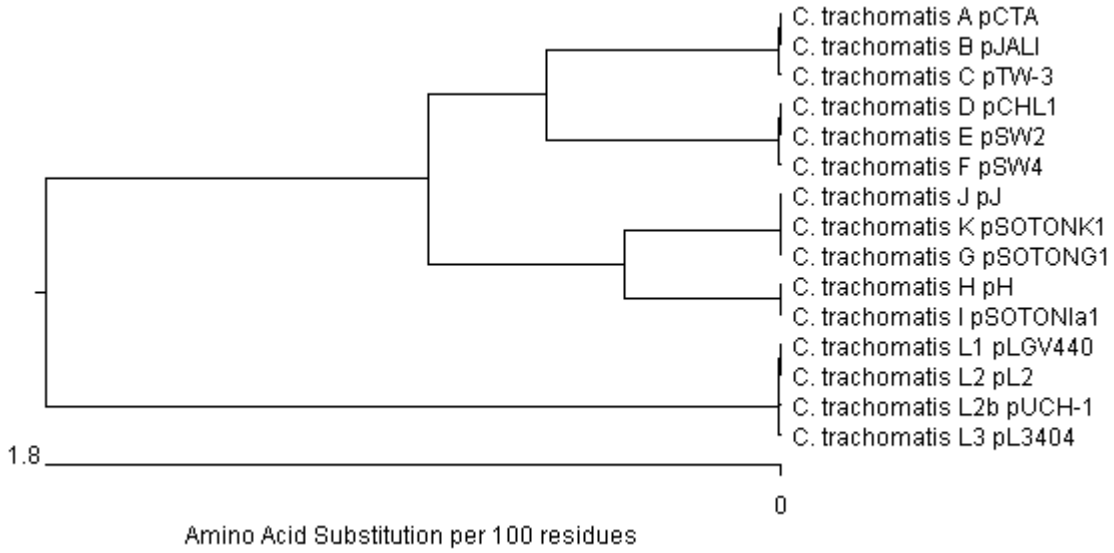


Table 1.3 Percentage differences and similarities in amino acids between PGP3 sequences between serovars A-K and serovars L1-L3 of *C. trachomatis.*

Percent Identity

Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
1		100.0	100.0	98.9	98.9	98.9	98.1	98.1	98.1	98.1	98.1	96.2	96.2	96.2	96.2	**1**
2	0.0		100.0	98.9	98.9	98.9	98.1	98.1	98.1	98.1	98.1	96.2	96.2	96.2	96.2	**2**
3	0.0	0.0		98.9	98.9	98.9	98.1	98.1	98.1	98.1	98.1	96.2	96.2	96.2	96.2	**3**
4	1.1	1.1	1.1		100.0	100.0	98.5	98.5	98.5	98.5	98.5	96.6	96.6	96.6	96.6	**4**
5	1.1	1.1	1.1	0.0		100.0	98.5	98.5	98.5	98.5	98.5	96.6	96.6	96.6	96.6	**5**
6	1.1	1.1	1.1	0.0	0.0		98.5	98.5	98.5	98.5	98.5	96.6	96.6	96.6	96.6	**6**
7	1.9	1.9	1.9	1.5	1.5	1.5		99.2	99.2	100.0	100.0	96.6	96.6	96.6	96.6	**7**
8	1.9	1.9	1.9	1.5	1.5	1.5	0.8		100.0	99.2	99.2	96.6	96.6	96.6	96.6	**8**
9	1.9	1.9	1.9	1.5	1.5	1.5	0.8	0.0		99.2	99.2	96.6	96.6	96.6	96.6	**9**
10	1.9	1.9	1.9	1.5	1.5	1.5	0.0	0.8	0.8		100.0	96.6	96.6	96.6	96.6	**10**
11	1.9	1.9	1.9	1.5	1.5	1.5	0.0	0.8	0.8	0.0		96.6	96.6	96.6	96.6	**11**
12	3.9	3.9	3.9	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5		100.0	100.0	100.0	**12**
13	3.9	3.9	3.9	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	0.0		100.0	100.0	**13**
14	3.9	3.9	3.9	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	0.0	0.0		100.0	**14**
15	3.9	3.9	3.9	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	0.0	0.0	0.0		**15**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	

1 C. trachomatis A pCTA
2 C. trachomatis B pJALI
3 C. trachomatis C pTW-3
4 C. trachomatis D pCHL1
5 C. trachomatis E pSW2
6 C. trachomatis F pSW4
7 C. trachomatis G pSOTONG1
8 C. trachomatis H pH
9 C. trachomatis I pSOTONIa1
10 C. trachomatis J pJ
11 C. trachomatis K pSOTONK1
12 C. trachomatis L1 pLGV440
13 C. trachomatis L2 pL2
14 C. trachomatis L2b pUCH-1
15 C. trachomatis L3 pL3404

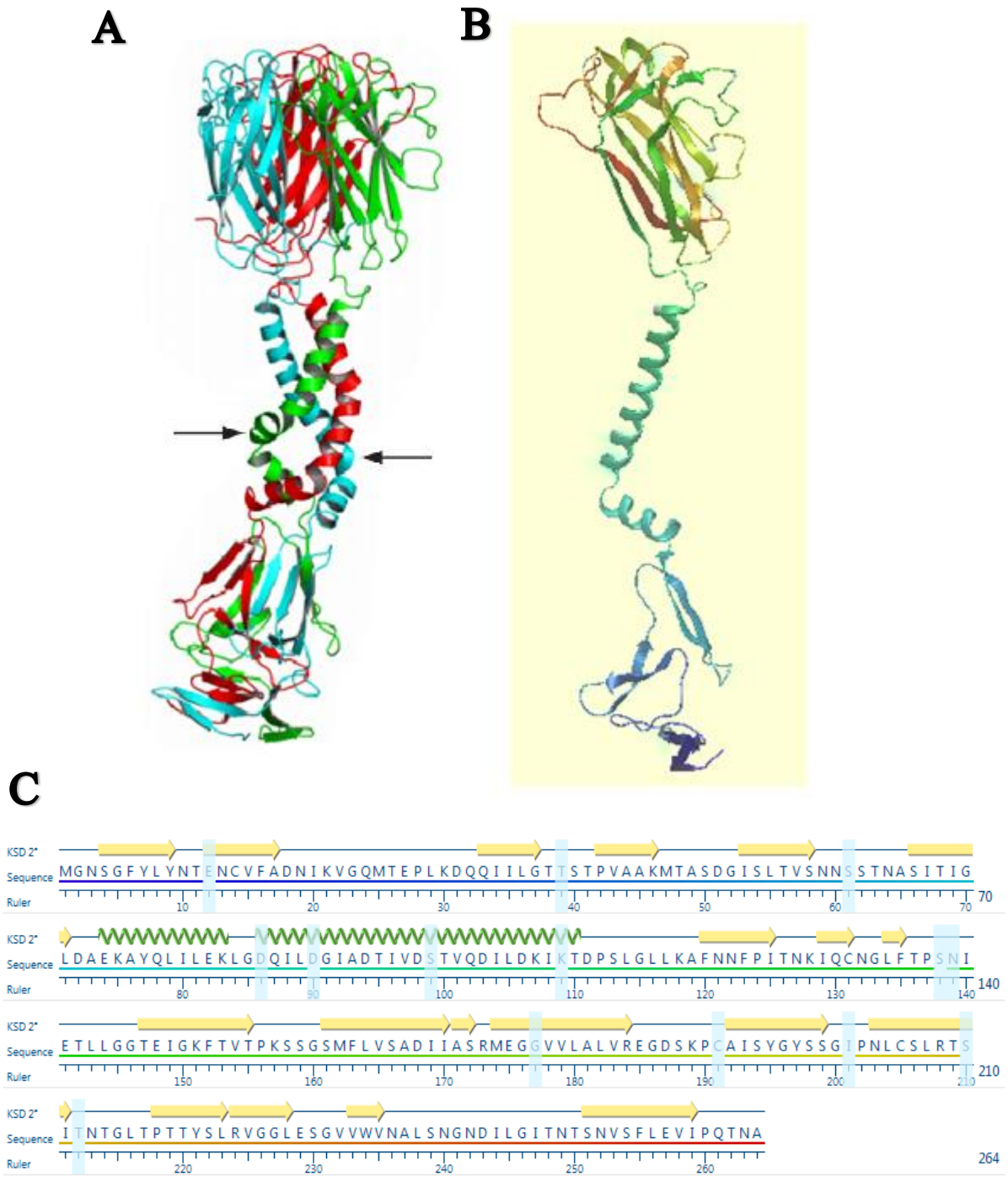
Table 1.4. Amino acid variation of monomeric, untagged PGP3 between serovars A-K and serovars L1-L3 of *C. trachomatis*. Sequences analysed using the MegAlign program from DNAStar Lasergene version 8.0 software package. Sequences for pH and pJ plasmids (James Hadfield, Sanger Institute) are not publicly available online. [Basic; acidic; hydrophobic; polar]

	Amino acid pos. no.	A/Har-12	Jali20	TW-3	G0/86	Sweden2	Sweden4	SotonG1	H	SotonIa1	J	SotonK1	L1/440/LN	L2/434/BU	L2b/UCH-1	L3/404
		pCTA	pJALI	Un-named	pCHL1	pSW2	pSW4	pSOTONG1	pH	pSOTONIa1	pJ	pSOTONK1	pLGV440	pL2	pUCH-1	pL3404
		EMBL: CP000052	EMBL: FM865438	Genbank: CP006946.1	Genbank: NC_001372	EMBL: FM865439	EMBL: FM865441	H603235	N/A	HE603236	N/A	HE603238	EMBL: X06707	EMBL: X07547	EMBL: AM886279	HE603228
1	12	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Glu	Gln	Gln	Gln	Gln
2	39	Thr	Thr	Thr	Thr	Thr	Thr	Lys	Thr	Thr	Lys	Lys	Thr	Thr	Thr	Thr
3	61	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Pro	Pro	Pro	Pro
4	86	Asp	Asp	Asp	Asp	Asp	Asp	Asn	Asn	Asn	Asn	Asn	Asp	Asp	Asp	Asp
5	90	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Gly	Gly	Gly	Gly
6	99	Asn	Asn	Asn	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
7	109	Lys	Lys	Lys	Lys	Lys	Lys	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
8	138	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Arg	Arg	Arg	Arg
9	139	Ser	Ser	Ser	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn
10	177	Gly	Gly	Gly	Gly	Gly	Gly	Gly	Ser	Ser	Gly	Gly	Gly	Gly	Gly	Gly
11	191	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Cys	Tyr	Tyr	Tyr	Tyr
12	201	Val	Val	Val	Ile	Ile	Ile	Val	Val	Val	Val	Val	Val	Val	Val	Val
13	210	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Arg	Arg	Arg	Arg
14	212	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ile	Ile	Ile	Ile
Theoretical Isoelectric point (pI)		4.39	4.39	4.39	4.39	4.39	4.39	4.46	4.37	4.37	4.46	4.46	4.64	4.64	4.64	4.64

1.13.2 Structural studies of PGP3

PGP3 consists of a 264 amino acid protein and the products polymerise to form an 84 kDa homotrimer (158). Galaleldeen *et al.* (158) determined the structure of PGP3 to be an elongated baton-like molecule with C-terminal domain folds similar to the TNF family of cytokines (figure 1.7A). The presence of glycine at position 85 (figure 17.A, arrow) acts as a pivot and results in a triple-helical coiled-coil with a right-handed twist between positions 73-84 and 86-111. The closest structural homologs were found to be lectin in *Burkholderia cepacia* and the surface protein BcIA in *Bacillus anthracis*, both of which are involved in cell adhesion. In figure 17C, amino acid variations between PGP3 derived from serovars A-K and L1-L3, as in Table 1.4, are highlighted and aligned with the PGP3 secondary structure. Previous reports (124, 125) have suggested that human antibody recognition is dependent on the native trimeric confirmation of PGP3. In these experiments, antibodies from humans infected with *C. trachomatis* only recognised the native conformation of PGP3 and none of the sera tested in the quoted studies reacted with the monomer of PGP3. However, these studies used very high serum dilutions of up to 1:50,000, so low levels of antibodies that may have been capable of binding the PGP3 monomer may not have been detected. Furthermore, this is not the first time that human antibody binding to monomeric PGP3 has been reported (75, 118, 123, 125, 159, 160). Monomeric binding to PGP3 in a western blot has also been reported using animal sera (160). Preferential antibody binding has also been observed in different mouse strains. In Western blot assays, the sera of infected C57BL/6N mice reacted with monomeric PGP3, whereas the sera of infected BALB/c mice reacted with trimeric PGP3 (155). Also using Western blot assays, Kari *et al.* (2013) (161) investigated the potential that human antibody recognition of PGP3 within chlamydial infected cell lysates was abolished by heat denaturation and found that recognition of denatured PGP3 was markedly reduced but not absent. However, it was not clear whether the antibody recognition was to monomeric or trimeric PGP3.

Figure 1.7. Structure of PGP3. [A] PGP3 as a trimer (derived from *C. trachomatis* serovar D)(158); **[B]** PGP3 as a monomer (derived from *C. trachomatis* serovar E); **[C]** Primary amino acid sequence of PGP3 (derived from *C. trachomatis* serovar E) aligned against the PGP3 secondary structure. Possible amino acid variation positions of PGP3 sequences derived from serovars A-K and L1-L3 are highlighted. Figures [B] and [C] were produced using the Protean 3D program from the DNAStar Lasergene version 14.0 software package with the Novafold add-on protein prediction software.



1.13.1 Studies of PGP3 in animal models

Although the expression of PGP3 is not essential for chlamydial growth, the importance of PGP3 in chlamydial infection has been demonstrated in several studies. Kari *et al.* (161) reported that immune responses to trachoma antigens, including PGP3, in a non-human primate model were found to change over the course of ocular infection. Antibodies to PGP3 peaked after 16 weeks post-infection and the generation of antibodies specific for PGP3 also correlated with the eradication of *C. trachomatis* infection. Experiments using urogenital isolates of *C. trachomatis* with plasmids in which CDS5 had been deleted revealed significantly reduced bacterial burdens in the genital tracts of mice, mimicking the properties of the plasmid-free (P^-) strains (135, 162). Similar to PGP4, PGP3 has also been revealed to induce hydrosalphinx in mice (162). Immunisation with PGP3 has shown reduced bacterial burdens in the lungs of infected mice, although protection was found to be due to CD4+ T cell-mediated immunity and not due to the presence of anti-PGP3 antibodies (84). PGP3 has also been shown to induce pro-inflammatory cytokines *in vitro* such as TNF-α, IL-1, IL-1β, IL-8, all of which contribute to the Th1-dominated immune response seen in chlamydial infections (162). These cytokines are known to induce the pro-inflammatory cytokine IFN-γ which is thought to increase the risk of ectopic pregnancy (63), as discussed in 1.7. However, these studies may not completely reflect chlamydial infection in humans as various levels of susceptibility and immune responses to PGP3 have been reported in different mouse strains (155, 162). Furthermore, PGP3 studies conducted using *C. muridarum* plasmids (e.g. pMoPn) may not reflect properties of PGP3 in *C. trachomatis* plasmids, as both proteins share an 82% amino acid identity (comparison of CDS5 from pSW2 (EMBL:FM865439) and pMoPn (Genbank: AE002162.1) using BLAST software, NCBI).

1.13.3 PGP3 as a marker in seroepidemiology

As discussed above, the unique properties of PGP3 make it an ideal antigen for detecting anti-*C. trachomatis* antibodies for use in ELISA formats for both diagnosis and seroprevalence. It has been used extensively in solid phase antibody detection systems and subsequently used for seroepidemiological analysis of *C. trachomatis* in populations. However, these ELISAs lack standardisation and there is significant variation between the assay methods, which include relevant controls, origin of the PGP3 and recombinant protein purification methods.

Most patients infected with *C. trachomatis* produce an antibody response to PGP3 (75, 118, 122, 160, 163). A number of studies incorporating PGP3 as a serological marker for chlamydial infection in an ELISA have already been published, as listed in Table 1.5. Whilst most of these PGP3 ELISAs have reported encouraging results, this lack of standardisation may question some *C. trachomatis* seroprevalence studies already undertaken. Wills *et al.* (2009) (118) were the first to produce a PGP3 ELISA using a very large cohort of well-characterised patient sera. When validating a new assay, large numbers of samples that have been exposed (positive) or unexposed (negative) to the antigen in question are recommended for a reliable statistical analysis. Wills *et al.* (2009) used 356 serum samples from patients collected one month after the diagnosis of chlamydia by NAAT and 747 serum samples from children aged under 13-years-old assumed to be negative to chlamydial antibodies, although small chances of past exposure through vertical transmission or sexual abuse were possible. Overall sensitivity and specificity of the PGP3 ELISA were found to be 57.9% and 97.6%, respectively, and intra- and interassay statistical analyses of the reproducibility of the assay were undertaken. Overall, the assay presented by Wills *et al*. (2009) appeared to be the most promising method of assessing seroprevalence of *C. trachomatis* in the population, as they later investigated (164-166).

There are a number of concerns regarding the methods used in the assay. Firstly, the PGP3 used in the study derives from the less common L1 serovar of chlamydia. This PGP3 structure differs by nine amino acids from PGP3 derived from the more common genital tract strain, E (126). This could account for minor differences in the structure of the antigen in the ELISA which may alter the overall sensitivity of the assay. Furthermore, Chen *et al.* (2010) reported that human antibody recognition of PGP3 is dependent on PGP3 being in its trimeric form (124). Although their reason is unclear, Wills *et al.* (2009) used DTT in their antigen solution. As DTT disrupts disulphide bonds in protein structures, the trimeric structure of PGP3 may be affected, as it is known that PGP3 deriving from the *C. trachomatis* pLGV440 plasmid contains three cysteine amino acids, whereas PGP3 deriving from pSW2 contains four. Furthermore, other studies have reported the human antibody binding of the PGP3 monomer, as listed in Table 1.6. The preferential binding of human antibodies to PGP3 monomers or trimers needs to be further investigated and taken into consideration when preparing the antigen for the assay, although it may not be possible to assess the structure of PGP3

when used in an ELISA format. It was noted that the Wills *et al.* (2009) ELISA used an antigen coating concentration of 20 ng/well which was later increased to 100 ng/well for the later seroprevalence studies. It is essential to coat the assay using the antigen at saturation, as calculated by clearly defined positive and negative controls. Here, increasing the antigen concentration would allow the detection of lower concentrations of antibodies to the antigen in sera, which would essentially create a new assay. However, the cut-off point of which a positive serum sample was defined was unaltered. This questions the reliability of the seroprevalence studies which were later undertaken using their PGP3 ELISA. Furthermore, the methods of background correction and assessment of potential false positive background in the assay are suboptimal. When used in an assay, even with the use of blocking agents sera contain non-specific by-products that cause false-positive background. The background of serum can be assessed by using non antigen coated wells or by using an unrelated protein as a negative control purified using the same method of the test antigen.

At least six other studies using PGP3 in an indirect ELISA have been described (75, 123, 160, 163, 167, 168). In 1994, Comanducci *et al.* (75) were the first to report the potential of sera collected from *C. trachomatis*-positive patients to react with PGP3 in ELISA and Western blot assays. Goodhew *et al.* (167) reported a high sensitivity and specificity of 91% and 98%, respectively. Goodhew *et al.* used a multiplex bead assay in which PGP3 is conjugated to agarose beads and incubated with serum samples and detection secondary antibodies, similar to an indirect ELISA. However, only a very small cohort of 11 serum samples from patients positive to chlamydia by PCR. Furthermore, a GST-PGP3 fusion protein was used in their ELISA but the study failed to mention the use of GST as a negative control protein. Bas *et al.* (123) also incorporated a PGP3 fusion protein in their ELISA instead containing a polyhistidine tag and reported a high sensitivity of 76% but a lower specificity of 77%. Similarly, they also failed to mention the use of a negative control protein. 31 serum samples in their negative cohort were described as ‘healthy donors’ and therefore had a chance of past chlamydia exposure which may account for the lower specificity seen in this assay. Donati *et al.* (160) and Ghaem-Maghami *et al.* (75) also reported sensitivities of 66% and 91.1%, respectively, although negative serum cohorts used in each study are not clearly defined and therefore the specificities of each assay are unclear.

The deployment of PGP3 using a sandwich ELISA method has also recently been undertaken. Li, *et al* (168) produced a sandwich ELISA using a monoclonal antibody to PGP3 and reported a high sensitivity and specificity of 92.1% and 100%, respectively. However, similar to Bas *et al.* their negative serum cohort consisted of 62 'healthy donors'. These donors were aged 22-65, and although confirmed seronegative to chlamydia by MIF, there is a chance that these sera contain anti-chlamydia antibodies which could be detected by a more sensitive assay. Furthermore, their motive of using a sandwich ELISA over an indirect ELISA is unclear. A sandwich ELISA is used when the antigen concentration of a sample is low or contaminated, and hence the use of a capture antibody in the ELISA would help to overcome this issue. Therefore, a sandwich ELISA would have no real advantage of increasing sensitivity by being able to detect lower levels of antibody in a sample. The description of the ELISA in the methods is also incoherent and it is therefore unclear to the reader how Li *et al.* managed to achieve a high sensitivity of 92.1%. As discussed, it may not always be possible to achieve such a high sensitivity figure as infected individuals may not always seroconvert and produce antibodies to PGP3. Furthermore, as mentioned above, some antibodies in serum samples may show preferential binding to the PGP3 monomer and/or trimer.

It is apparent that with each study undertaken that there is no standardisation between these PGP3 ELISAs. However, there are basic measures to consider when preparing a new serological assay. One of the most important considerations is to have a large cohort of clearly defined exposed and unexposed serum samples that have been defined by at least one other method. Whilst this may not always be possible, it is therefore essential to use serum from immunised and/or infected animals as a positive control for use in assay each run. Controls must also be used for the initial assay optimisation of the assay when determining the concentration of antigen saturation. Negative control proteins should also be used, in particular when a fusion protein is being used in the assay. This would exclude any potential from false positive background from unwanted cross reaction with the fusion tag. A negative control protein is also particularly useful to assess false-positive control caused from non-specific products in the sera. Furthermore, false positive background can be reduced by finding the most effective blocking agent to use in that assay that is most suitable for the antigen and serum. Finally, all assays need to demonstrate their reproducibility. Not all studies using PGP3

in their ELISAs have demonstrated their reproducibility and those that have shown a great deal of variation between the methods used. Ideally, a number of sera would need to be assessed multiple times within the assay and be repeated on a number of different days to produce accurate reproducibility results. Additionally, the assay should also be repeated by another operator to show inter-operator reproducibility. Collectively, a sensitive, accurate and reliable serological assay could be produced if these measures were applied to an ELISA incorporating PGP3.

Table 1.5. Summary of assays using PGP3 to detect anti-chlamydia antibodies in human and animal sera.

PGP3 serovar/ strain	Type of assay	Expression vector/ tag	Cleaved protein	Native/ denaturing purification	Ag coating concentration	Source of sera/ dilution	No. of positive controls	No. of negative controls	Sensitivity^/ seropositivity+(%)	Specificity (%)	Blank correction method	Reference
D[159]	Multi-plex bead assay	pGEX-6P/ GST (N-term)	No	Native&	120 µg Ag per 1.25 x 10^6 beads	Human/ 1:400	11[de]	112[b]	91.0^	98.0	N/S	Goodhew *et al* (167)
						Human/ 1:400 (167)	575[abc]	N/A	33.8+ *	N/A	N/S	Martin *et al*., (169)
							1474[b]	N/A	7.5+	N/A	N/S	West *et al.* (170)
							1124[b]	N/A	1.5+	N/A	N/S	Pant *et al.* (171)
LGV440	Indirect ELISA	pGEX-4T-1/ GST (N-term)	Yes	Denaturing? & 5 mM DTT added	20 ng/well	Human/ 1:100	356[de]	747[b]	57.9^	97.6	Subtracted OD of Ag non-serum coated wells from Ag serum coated wells	Wills *et al*., (118)
					100 ng/well	Human 1:100 (118)	4732[a] females	80	6.0-26.0+	N/A		Horner *et al*., (165)
					20 ng/well (118)		164[d]	N/A	74.0+	N/A		Horner *et al*., (164)
LGV440[118]	Double antigen ELISA	pGEX-4T-1/ GST (N-term)(118)	Yes	Denaturing? & 5 mM DTT added (118)	N/S	Human/ 1:4	342[de]	505[b]	82.9^ females 54.4^ males	97.8	N/S	Horner *et al.* (172)
D	Indirect ELISA	pQE-60/ His (C-term)	No	Native	100 pmol/ml	Human 1:100	45[cde]	31[a]	53.0^	80.0	Subtracted OD of serum non-Ag coated wells from Ag serum coated wells	Bas *et al*., (123)
						Human 1:100(123)	28[cde]	56[a]	57.0+	89.0		Bas *et al*., (122)
							17[def]	20[a]	59.0	90.0		Bas *et al*., (173)

Table 1.5 continued.

PGP3 serovar/ strain	Type of assay	Expression vector/ tag	Cleaved protein	Native/ denaturing purification	Ag coating concentration	Source of sera/ dilution	No. of positive controls	No. of negative controls	Sensitivity^/ seropositivity+(%)	Specificity (%)	Blank correction method	Reference
C. psittaci, *C.trachomatis* (strains N/S) **(174)**	Indirect ELISA	*C.psittaci* pET23b/ His (C-term); *C.trachomatis* (75)	No	Denaturing	600 ng/well	Duck, Pidgeon, Cat, Pig, Human/ all 1:100	50[f]; 454[h]	20 (background N/S); None	66.0+; 52.2+	N/S	N/S	Donati *et al*., (160)
D	Indirect ELISA	pT7-7	N/A	Native	200 ng/well	Human/ N/S	110[g]	50[g]	11.10-83.3^	100%	N/S	Comanducci *et al*., (75)
D **(75)**	Indirect ELISA	pT7-7 (75)	N/A	Native	600 ng/well	Human/ 1:4 and 1:20	62[de]	Quantity & background N/S	95.1^	N/S	N/S	Ghaem-Maghami *et al*., (163)

N/A = not applicable; S/T = not stated; [a] = healthy adult donors (no chlamydia symptoms present; not tested for chlamydia by NAAT or MIF); [b] = healthy child donors (no chlamydia symptoms present; not tested for chlamydia by NAAT or MIF) [c] = chlamydia symptoms present (PID/ urethritis) but not diagnosed by NAAT or MIF; [d] = females diagnosed positive/negative for chlamydia by NAAT; [e] = males diagnosed positive/negative for chlamydia by NAAT; [f] = positive for chlamydia by isolation; [g] = seronegative/seropositive by MIF to chlamydial EBs; [h] = from non-human animals (avian, feline, porcine); [&] = assumed naturing/denaturing purification according to methods although not formally stated; * = seroprevalence of antibodies to PGP3 as a single antigen in the assay is not stated (stated seroprevalence percentage is combined figure of PGP3 and CT694).

Table 1.6. Summary of western blotting assays using PGP3 to detect anti-chlamydia antibodies in human and animal sera.

PGP3 serovar/ strain	Expression vector/ tag	Cleaved protein	WB detection method	Source of sera/ dilution	No. of positive controls	No. of negative controls	Sensitivity[^]/ seropositivity[+] (%)	Specificity (%)	Monomer of PGP3 detected?	Trimer of PGP3 detected?	Reference
D	pEX3A/ MS2 peptide	No	Colori-metric (AP)	Human/ 1:100	32[g]	20[a]	78.1[^]	100.0	Yes	N/A	Coman-ducci *et al.*, (75)
D	pQE-60/ His (C-term)	No	Colori-metric (AP)	Human/ 1:100	Pool of 22[de]	N/S	N/S	N/S	Yes	N/S	Bas *et al.*, (123)
D	*C.trachoma-tis* cell lysates	N/A	ECL (HRP)	Human/ 1:1000	Pool of 99[de]	Pool of 8 (Background N/S)	N/S	N/S	Yes	N/S	Wang *et al.*, (159)
C. psittaci, *C.trachoma-tis* (strains N/S) **(174)**	*C.psittaci* pET23b/His (C-term); *C.trachoma-tis* (75)	No	ECL (HRP)	Human/ 1:100; Animal/ 1:100	50[f]; 454[h]	20 (back-ground N/S); None	66.0[^]; 58.3[^]	N/S	Yes*	N/S	Donati *et al.*, (160)
LGV440	pGEX-4T-1/ GST (N-term)	Yes	ECL (HRP)	Human/ 1:100	1 sample from 356[de]	N/S	N/A	N/A	Yes	N/S	Wills *et al.*, (118)

Table 1.6 continued.

PGP3 serovar/ strain	Expression vector/ tag	Cleaved protein	WB detection method	Source of sera/ dilution	No. of positive controls	No. of negative controls	Sensitivity[^]/ seropositivity[+] (%)	Specificity (%)	Monomer of PGP3 detected?	Trimer of PGP3 detected?	Reference
D	pGEX/ GST (N-term)	No	ECL (HRP)	Human/ 1:4000-1:10^6	Pool of 15[de]	Pool of 8 (Background N/S)	N/A	N/A	Yes (1:4000 dilution only)	N/S	Li *et al*., (125)
D	pGEX-6P-2/ GST (N-term)	Yes	ECL (HRP)	Human/ 1:2000-1:50,000	Quantity N/S[de]	Pool of 8 (Background N/S)	N/A	N/A	No	Yes (all serum dilutions)	Chen *et al*., (153)
C.murida-rum Nigg	P6HisF-11d pET/ His	Yes	Colori-metric (HRP)	Mouse (BALB/c, C57BL/6)/ 1:50	Pooled[i], quantity N/S	Pooled[i], quantity N/S	N/A	N/A	Yes – mice strain-dependent (C57BL/6)	Yes – mice strain-dependent (BALB/c)	Mosolygo *et al*., (155)
				Mouse (C57-BL/6) / 1:50	Pooled[j], quantity N/S	Pooled[j], quantity N/S	N/A	N/A	Yes	N/A	Mosolygo *et al*., (175)

N/A = not applicable; S/T = not stated; [a] = healthy adult donors (no chlamydia symptoms present; not tested for chlamydia by NAAT or MIF); [b] = healthy child donors (no chlamydia symptoms present; not tested for chlamydia by NAAT or MIF) [c] = chlamydia symptoms present but not diagnosed by NAAT or MIF (PID/ urethritis); [d] = females diagnosed positive or negative for chlamydia by NAAT; [e] = males diagnosed positive or negative for chlamydia by NAAT; [f] = positive for chlamydia by isolation; [g] = seronegative or seropositive by MIF to chlamydial EBs; [h] = from non-human animals (avian, feline, porcine). [i=] sera from infected/non-infected mice; [j] = sera from immunised/non-immunised mice; * = n/s but methods describe denaturation and detection of PGP3

1.14 AIMS AND HYPOTHESES

1.14.1 Gaps in knowledge

No systematic investigation of the seroprevalence of *C. trachomatis* in the under 16s population in England has been conducted, despite evidence of chlamydial diagnoses and increasingly earlier sexual debut. As patients under 16-years-old are not actively recruited for chlamydial screening, this data will provide an insight into the need for chlamydial screening and new interventions in children.

The National Chlamydia Screening Programme (NCSP) was launched in 2002 to offer opportunistic chlamydial screening to young people aged 15-25-years-old in England. Chlamydia is most commonly diagnosed in this age group and over 138,000 chlamydial diagnoses were made in 2014 (see Table 1.1 and (89)). However, the justification for the cut-off at age 15 is unclear and there is evidence of increasingly earlier and unprotected sexual debut (98, 176-178). Furthermore, 15-year-olds are often only tested under special circumstances and parental notification is often encouraged in accordance with the Fraser Guidelines (109). Therefore, diagnoses made in the under 16s are not representative of the overall population. One study found nearly 30% of the 16 to 24-year-old males and females they surveyed experienced their first sexual encounter before age 16 in comparison to only 4% of women that were aged 65 to 74-years-old (98). In 2011, an English survey reported that 1.9% experienced non-consensual intercourse and 8.3% experienced some form of sexual abuse before age 16 (178). Another study found that 29.9% of men and 25.6% of women reported having their first sexual experience before age 16 (176). Furthermore, over 14% of respondents reported first sexual intercourse at age 13-14-years-old. Alarmingly, the same study reported 17.9% of males and 21.7% of females who had their first sexual encounter at age 13-14-years-old reported no use of any contraception, compared to 9.8% and 10% of 15-year-old males and females, respectively. This is reflected in the high teenage conception rate. Although the rate has fallen over the last two decades, the conception rate for 15-17-year-olds in England in 2011 remains one of the highest in Europe at 30.7conceptions per 1000 women (179). In 2013, over 21,000 young people under 15 in England were screened for chlamydia infection (89). 1,100 (5.4%) of these tests were diagnosed as positive, representing 0.2% of the overall population for young people under 15.

1.14.2 Serological tests are the most appropriate tool to address these gaps in knowledge

As the NCSP relies on opportunistic testing, and because many cases of chlamydia are asymptomatic (48), diagnoses made by NAATs are an underrepresentation of the prevalence of chlamydia in the under 16s population. Therefore, a seroprevalence study would be the most appropriate method to determine the prevalence of chlamydia in the under 16s population, as a majority of patients infected with chlamydia produce antibodies to chlamydial antigens (75, 118, 123, 160, 163, 167, 168). Some incidental evidence of the seroprevalence of *C. trachomatis* in the under 16s population has been described. Wills *et al.* (118) used over 700 serum samples from children aged 2-13-years-old as negative controls for their PGP3 ELISA and 3.3% were found to be positive for *C. trachomatis* antibodies by MIF. Goodhew, *et al.* (167) also conducted a serological study using sera from a non-endemic children cohort (<6-years-old) as presumed negative controls to a PGP3 ELISA study. Of the 122 sera assayed, 3 (2.4%) were found to be positive to PGP3. Horner *et al.* (165) reported the seroprevalence of chlamydia in female 17-18-year-olds in England to be 13.24%. Despite this evidence of chlamydial prevalence and sexual activity in the under 16s, a crucial gap in seroepidemiological knowledge still exists for the under 16s population.

1.14.3 Hypotheses

Due to evidence of early sexual debut in the under 16s, we hypothesise that the seroprevalence of chlamydia in the under 16s is likely to be similar to the 13.24% rate reported by Horner *et al.* (165) in the 17-18-year-olds. Our null hypothesis is that the seroprevalence rate of chlamydia in the under 16s is similar to the 3.3% rate reported by Wills *et al.* (118).

1.14.4 Approach

In 2007, Jit *et al.* (180) conducted a population-based study on the seroprevalence of human papillomavirus (HPV) in young women aged 10-29-years-old using age-stratified serum samples collected from laboratories in England. Since this study, the seroepidemiology unit (SEU) at Public Health England (PHE) have continued to collect serum samples from laboratories across England as part of their serum bank collection for further seroprevalence studies. The SEU also allows these serum samples to be used

by other research groups for seroepidemiological research. Our overall aim is to use these samples to determine the seroprevalence of chlamydia in the under 16s in England. To determine the seroprevalence rate of chlamydia in the under 16s, the prevalence of anti-PGP3 antibodies in patient serum samples will be measured using an ELISA based on recombinant PGP3. Previous PGP3 ELISAs have been described (75, 118, 123, 160, 163, 167, 168). However, as discussed in 1.13.3, these methods vary in the type of serological assay used, assay method and the number and types of controls used to optimise and validate the assays. These ELISAs also vary by the *C. trachomatis* serovar derivation of PGP3. Wills *et al.* (118) used recombinant PGP3 derived from the less common L1 serovar in their ELISA. This varies from the urogenital derived PGP3 by nine amino acids which could account for structural differences affecting the sensitivity of the assay (see Table 1.3). For our ELISA, we therefore aimed to use PGP3 derived from a common urogenital tract strain of *C. trachomatis*, serovar E, to produce a sensitive, specific and reproducible ELISA to detect anti-PGP3 antibodies in patient serum samples. PGP3 derived from urogenital *C. trachomatis* serovars D-F share a 100% amino acid identity and are the most prevalent genital tract strains worldwide (30-32).

1.14.5 Specific aims and research goals

The specific aims of this project are to:

- Develop a sensitive and specific PGP3 ELISA to detect anti-PGP3 antibodies in patient sera as an indicator of prior *C. trachomatis* exposure.
    - Express and purify recombinant PGP3 derived from *C. trachomatis* serovars E (pSW2) and L1 (pLGV440) to use as candidate detection antigens
    - Develop and optimise indirect ELISAs and Western blots to detect anti-PGP3 antibodies in patient sera
- Evaluate the PGP3 ELISAs using sera collected from patients with a known *C. trachomatis* status, as diagnosed by NAAT
- Apply the evaluated ELISA to detect anti-PGP3 antibodies in a significant group of sera collected from children under 16 from England and use the data to determine the seroprevalence of *C. trachomatis* in this population.

CHAPTER TWO: MATERIALS AND METHODS

2.1 CHEMICALS AND SOLUTIONS

2.1.1 Water

An Elix Millipore (EMD Millipore) system was used to produce deionised water (dH_20) using reverse osmosis. Ultra high quality (UHQ) dH_2O was prepared using the Barnstead NANOpure Diamond ultrapure water system. This system further deionises and purifies dH_2O using a UV lamp, an ultrafilter and a 0.2µm filter to produce DNase and RNase-free UHQ. For nucleic acid manipulation, sterile Gibco DNase and RNase-free H_20 was used and purchased from ThermoFisher Scientific.

2.1.2 Sterilisation

Non-sterile pipette tips, media, bulk solutions and glassware were autoclaved at 120°C and 15 psi for 20 minutes. Smaller volumes of media or other solutions were autoclaved at 120°C and 15 psi for 15 minutes using a Tefal Clipso benchtop pressure cooker. For heat-sensitive solutions (e.g. antibiotics), a 0.2µm filter (Millipore) was used for sterilisation.

2.1.3 Buffers, reagents and solutions

Buffers, reagents and other solutions were commercially sourced and ready-made, if available. If required, buffers, reagents and solutions were prepared using UHQ dH_2O and filtered using a 0.2µm syringe filter (Millipore).

2.1.4 Plastic and glassware

Sterile plastic ware (e.g. 1.5 ml disposable polypropylene tube (Sarstedt Inc, UK), universals, bijoux and stripettes) were purchased from various suppliers and autoclaved at 15 psi for 30 minutes, if required. Non-sterile pipette tips were purchased from Alpha Laboratories Ltd (Eastleigh, Hampshire) and autoclaved at 15 psi for 30 minutes. Sterile, DNase and RNase-free filter pipette tips were purchased from Sarstedt Inc or Axygen (Thistle, UK). Nuclease-free 200 µl PCR tubes were purchased from Axygen (Thistle, UK). Sterile plastics such as universals and petri dishes (90 mm diameter) were purchased from Sterilin (UK).

2.2 BACTERIAL GROWTH MEDIUM AND SOLUTIONS

2.2.1 Luria Bertani (LB) medium

LB medium was used for the growth of *Escherichia coli* strains (Table 2.1). Tryptone (Difco, Becton, Dickinson (BD) and Company, USA) (10 g/L), yeast extract (Difco, BD, USA) (5 g/L) and NaCl (5 g/L) were dissolved in UHQ dH_20 and adjusted to pH 7.5 with 5M NaOH. The medium was autoclaved (2.1.2) then stored at room temperature or 4°C.

2.2.2 LB agar

LB agar plates were used for the growth and maintenance of *E. coli* strains. 1.5% (w/v) agar (Difco, BD, USA) was added to LB media (2.2.1), autoclaved (2.1.2), cooled to 50°C and poured into sterile petri dishes. The agar plates were allowed to set at room temperature and surfaces were air-dried in a Class II laminar flow hood. Unused plates were inverted and stored at 4°C for a maximum of two weeks and air-dried before use.

2.2.3 50% Glycerol

50% glycerol was used for long term storage of bacterial suspensions. 50% (v/v) glycerol in UHQ dH_20 was autoclaved (2.1.2) in 10 ml aliquots and added 50% (v/v) to growth suspensions in LB (2.3.1). 1 ml aliquots of prepared suspensions were stored in 1.2 ml cryo-vial tubes (Alpha Laboratories Ltd) and stored at -70°C.

2.2.4 Super Optimal Broth (SOB) medium

SOB medium was used for the growth of *E. coli* during recombinant protein expression. Tryptone (Difco, BD, USA) (20 g/L), yeast extract (Difco, BD, USA) (5 g/L), NaCl (0.5 g/L) and KCl (0.192 g/L) were dissolved in UHQ dH_20 and adjusted to pH 7 with 5M NaOH. The medium was autoclaved (2.1.2) then stored at room temperature or 4°C. Before use, 100 µl of 1M $MgCl_2$ was added per 10 ml of SOB medium.

2.2.5 SOC medium

SOC medium was used for the growth and recovery of *E. coli* following competent cell transformation. SOB medium was prepared (2.2.4), autoclaved, cooled to 50°C and 20 ml of 1M glucose was added per litre of medium. Final SOC medium was stored at room temperature or 4°C.

2.2.6 Antibiotics

Ampicillin (sodium salt, Sigma-Aldrich) was dissolved in UHQ dH_20 to 50 mg/ml. Chloramphenicol (Sigma-Aldrich) was dissolved in 100% ethanol to 35 mg/ml. All antibiotics were sterilised through a 0.2μm syringe filter (Millipore) and stored at -20°C in 1 ml aliquots. Ampicillin and chloramphenicol were added to LB medium (2.2.1) or molten LB agar at 50°C (2.2.2) at final concentrations of 50 μg/ml or 35 μg/ml, respectively, for the growth of ampicillin or chloramphenicol resistant *E. coli*.

2.3 BACTERIA AND PLASMIDS

2.3.1 Growth and storage

The strains of *E. coli* and plasmid vectors used in this work are listed in Tables 2.1 and 2.2. Frozen culture stocks (2.2.3) were used to produce working stocks on LB agar with antibiotics (if required) and were stored at 4°C. Liquid cultures of *E. coli* were grown in LB broth (2.2.1) for 16 hours at 200 rpm on a shaker at 37°C. Suspensions were added to 50% sterile glycerol (2.2.3) for long-term storage. Overnight starter cultures of *E. coli* strains were prepared by inoculating 10 ml LB broth with a colony and antibiotics (if required) and grown for 16 hours at 200 rpm on a shaker at 37°C (2.2.1).

2.3.2 Preparation of competent E. coli

For the transformation of *E. coli*, all reagents and materials were kept at 4°C. A 10 ml overnight starter culture (2.3.1) of the recipient *E. coli* strain was grown overnight at 37°C. A 1 ml aliquot of the starter culture was used to inoculate 100 ml LB medium (2.2.1) and was incubated at 37°C on a 200 rpm shaker until OD_{600} absorbance value reached 0.4 on a spectrophotometer. The culture was immediately placed on ice for 20 minutes and all subsequent procedures were performed at 4°C. Cells were pelleted by centrifugation at 3000 x *g* for 15 minutes and the supernatant was discarded. The bacterial pellet was resuspended in 20 ml of ice-cold 0.1M $CaCl_2$ and kept on ice for 20 minutes. The suspension was centrifuged for 15 minutes at 2000 x *g*. The supernatant was discarded and the pellet was resuspended in 5 ml 0.1M $CaCl_2$ with 15% (v/v) glycerol. Cells were pelleted once more by centrifugation for 15 minutes at 1000 x *g* and the supernatant was discarded. Cells were resuspended in 2 ml 0.1M $CaCl_2$ with 15% (v/v) glycerol and stored at -70°C in 100 μl single-use aliquots. Cells were thawed on ice prior to transformation.

Table 2.1 *Escherichia coli* strains and features.

Strain	Genotype	Resistance	Reference
DH5α	F^-, ø80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *recA*1,*endA*1, *hsdR*17(rK^-, mK^+), *phoA*, *supE*44, λ–, *thi*-1, *gyrA*96,*relA*1	None	Hanahan, 1983
BL21 (DE3) pLysS	F-, *omp*T, *hsd*S_B (r_B-, m_B-), *dcm*, *gal*, λ (DE3), pLysS, Cm^r	Chlor-amphenicol	Novagen

Table 2.2 Plasmid vectors and features

Plasmids	Size (bp)	Features	Resistance	Reference
pRSET-A	2,900	Cloning vector. T7 polymerase promoter. Expressed protein is fused with a N-terminal polyhistidine (x6) and Xpress Epitope tag of approximately 7 kDa	Ampicillin	Invitrogen
pGEX-4T-1	4,900	Cloning vector. Expressed protein is fused to an N-terminal 26 kDa glutathione s-transferase (GST). Expressed protein is cleaved from tag by thrombin (-4T-1) or Prescission Protease (-6P-1)	Ampicillin	GE Healthcare
pGEX-6P-1	4,900		Ampicillin	GE Healthcare

2.3.3 Transformation

Ice-cold DNA (10-100 ng) was added to ice-thawed competent *E. coli* cells (2.3.2) and kept on ice for 20 minutes. Cells were heat shocked at 42°C for 45 seconds to enable the uptake of DNA and allowed to recover on ice for a further 2 minutes. 1 ml of SOC medium (2.2.5) was added to the transformed cells and incubated at 37°C on a shaker at 200 rpm for one hour to confer antibiotic resistance from plasmid DNA taken up by the competent *E. coli* cells. Cells were pelleted by centrifugation at 3000 x *g* for 10 minutes at room temperature (20°C). Supernatant was discarded and cells were resuspended in 100 µl of LB medium (2.2.1). Cells were plated onto LB agar plates (2.2.2) containing the appropriate selective antibiotic and incubated overnight at 37°C.

2.4 ANALYSIS OF NUCLEIC ACIDS

2.4.1 Materials

DNase, RNase and protease-free 50x stock of TAE buffer (Tris(24%)-Acetate(5%)-EDTA(<2%), pH 8.2-8.4, Fisher Scientific) prepared at a 1x working dilution was used as the electrophoresis buffer for horizontal agarose gels. Orange G loading dye (x6, Sigma-Aldrich) was used as the sample loading buffer to track the migration of samples through the gel as it migrates faster (50 bp) than other dyes such as bromophenol blue (300 bp). Sybr Safe DNA gel stain (10,000x, Invitrogen) was added to molten agarose gels (2.4.2) and was used to visualise DNA in the agarose gels as it is a safer alternative to hazardous ethidium bromide staining also used for DNA visualisation.

2.4.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation, quantification and purification (2.5.2) of DNA fragments. 1-1.5% (w/v) agarose was dissolved in 1x TAE buffer by heating to 100°C in a microwave until completely dissolved. The molten agarose was cooled until 50°C and Sybr Safe (2.4.1) was added to a final concentration of 1x. The molten agarose was poured into a gel mold with a comb and allowed to set at room temperature for 20-30 minutes. The comb was removed and the gel was submerged in 1x TAE buffer. DNA samples containing 1x Orange G loading dye and 5 µl of Hyperladder I or II (Bioline) were loaded into the wells of the gel. Hyperladder markers are used to estimate the size, quality and quantity of separated DNA bands. Prepared

and loaded agarose gels were electrophoresed at 90 volts until there was sufficient separation and distinction between DNA bands (approximately 1 hour).

2.4.3 Visualisation and quantification of nucleic acids

Bands of DNA separated by agarose gel electrophoresis (2.4.2) were visualised at 302 nm using a UV transilluminator (UVP BioDoc-It System) and photographs were taken using Polaroid 667 film. Concentrations and sizes of plasmid DNA or PCR amplification products were estimated by comparison with bands of known concentration using Hyperladder I (200 to 10,000 bp) or II (50 to 2000 bp) markers (Bioline) (10 µl contained 30-200 ng/band). Alternatively, concentrations were measured using a Nanodrop 1000 spectrophotometer (Thermo Scientific) using the supplied software package. Blank measurements were taken using the relevant buffer that samples were stored in. An absorbance ratio of ~1.8 was accepted as pure for DNA at wavelengths 260nm and 280nm.

2.5 ISOLATION AND PURIFICATION OF NUCLEIC ACIDS

2.5.1 Wizard Plus SV Minipreps DNA Purification System

Small scale purification of plasmid DNA for manipulation (2.7) was achieved using a miniprep kit (Promega). Bacteria in 10 ml LB medium (2.2.1) with antibiotics (if required) were incubated overnight (2.3.1) and pelleted by centrifugation for 10 minutes at 10,000 x *g*. Supernatants were discarded and pellets were resuspended in 250 µl of Cell Resuspension Solution by vortex. 250 µl of Cell Lysis Solution was added and the solution was inverted four times and incubated for up to 5 minutes until the suspension cleared. To inactivate endonucleases, 10 µl of Alkaline Protease Solution was added and the solution was inverted four times and incubated for 5 minutes. 350 µl of Neutralisation Solution was added and the solution was inverted four times and centrifuged at 14,000 x *g* for 10 minutes. Approximately 850 µl of cleared lysate was transferred to a 2 ml Spin Column and centrifuged at 14,000 x *g* for 1 minute. Flow through was discarded and 750 µl od prepared Column Wash Solution was added to the Spin Column and centrifuged at 14,000 x *g* for 1 minute. Supernatant was discarded and 250 µl was added to the Spin Column and centrifuged at 14,000 x *g* for 2 minutes. The Spin Column was transferred to a new collection tube and 100 µl of nuclease-free water (2.1.1) was added and centrifuged at 14,000 x *g* for 1 minute to elute the plasmid DNA.

Purified plasmid DNA was analysed by agarose gel electrophoresis (2.4.2) and stored at -20°C.

2.5.2 Wizard SV Gel and PCR Clean-Up System

Purification of PCR products and DNA from agarose gels was achieved using a purification kit (Promega). DNA separated by agarose gel electrophoresis (2.4.2) was removed with a scalpel and weighed. Membrane Binding Solution was added at 10 µl per 10 mg of agarose gel slice and incubated at 50-60°C until the gel slice was completely dissolved (approximately 10 minutes). For PCR amplification products, an equal volume of Membrane Binding Solution was added. Dissolved gel slices or prepared PCR products were added to an SV Minicolumn assembly and incubated at room temperature for 1 minute. The Minicolumn was centrifuged at 16,000 x *g* for 1 minute and the supernatant was discarded. 750 µl of prepared Membrane Wash Solution was added and the Minicolumn was centrifuged for 2 minutes at 16,000 x *g*. Supernatant was discarded and 500 µl Membrane Wash Solution was added to the Minicolumn and centrifuged for 5 minutes at 16,000 x *g* to evaporate any residual ethanol. The Minicolumn was transferred to a new collection tube and 50 µl of nuclease-free water (2.1.1) was added and incubated at room temperature for 1 minute. Purified DNA and PCR products were eluted by centrifugation at 16,000 x g for 1 minute. Purified DNA and PCR products were analysed by agarose gel electrophoresis (2.4.2) and stored at -20°C.

2.5.3 STET (sucrose-Triton-EDTA-Tris)

Analysis and screening a large number of transformants (2.3.3) was achieved using STET buffer (8% sucrose, 5% Triton-X, 50 mM EDTA, 50 mM Tris pH 8.0) to purify plasmid DNA from transformed *E. coli* cultures. This is a cheaper and alternative method to the Wizard Plus SV Minipreps DNA Purification System (2.5.1). Single transformants were sub-cultured further onto LB agar plates (2.2.2) and incubated overnight at 37°C. An inoculating loop of *E. coli* from a sub-cultured plate was resuspended in 100 µl of STET buffer. 8 µl of lysozyme (10 mg/ml) was added to each sample and incubated at room temperature for 5 minutes. Cell lysis was achieved by boiling the samples at 100°C for 40 seconds. Cell debris was removed by centrifugation at 14,000 rpm for 10 minutes. Supernatants containing plasmid DNA were transferred to new 1.5 ml tubes and precipitated with an equal volume of isopropanol for 1 hour at -

20°C. Precipitated DNA was recovered by centrifugation at 14,000 rpm for 5 minutes, washed with 80% (v/v) ethanol and centrifuged as before. Excess ethanol was evaporated under a vacuum for approximately 15 minutes and DNA pellets were resuspended in DNase and RNase free water (2.1.1). A 5 µl aliquot was used for analysis by agarose gel electrophoresis (2.4.2).

2.6 POLYMERASE CHAIN REACTION

2.6.1 Materials

The thermostable properties of *Thermus aquaticus* (*Taq*) DNA polymerase have been exploited in the development of a DNA amplification procedure using PCR (181). Phusion Flash High Fidelity PCR Master Mix (x2, New England Biolabs) was used to amplify DNA by PCR. This master mix contains a high fidelity *Taq* DNA polymerase and contains all necessary reagents required for PCR, including an optimised reaction buffer and $MgCl_2$, with the exception of the DNA template and primers. This master mix is accurate and rapid and possesses an error rate approximately 25-fold lower than that of *Taq* DNA polymerase. DNA products amplified using this master mix were used for DNA manipulation and cloning (2.7). DNA products amplified using BioMix Red PCR master mix (Bioline) were used to screen possible recombinant plasmids from transformants (2.3.3) for DNA inserts. This master mix contains all reagents necessary for PCR, with the exception of DNA templates and PCR primers, and contains Taq DNA polymerase. BioMix Red PCR master mix also contains an inert red dye that allows for direct loading of samples onto an agarose gel (2.4.2) for analysis. DNA samples amplified by Phusion Flash High Fidelity PCR master mix were added to Orange G loading dye (2.4.1) before analysis by agarose gel electrophoresis. All samples were prepared using Gibco DNase and RNase-free UHQ dH_20 (Life Technologies) (2.1.1) under a PCR cabinet workstation equipped with a UV light.

2.6.2 Primer design

Custom oligonucleotides used for the amplification of DNA by PCR were synthesised by Eurogentec, UK. Custom primers were synthesised with restriction sites (2.7.1) to facilitate ligation (2.7.3) of amplified DNA products into the appropriate plasmid vectors. Custom primers were designed using DNAStar Lasergene version 8.0 software package (Lasergene, Madison, WI, USA) (2.13) and met the following criteria: primers should not contain palindromic sequences; the primers should have similar T_m values;

primers should not be complementary; primers should not dimerise; and primers should be between 17-40 bp in length.

2.6.3 PCR conditions

Samples were amplified in a Veriti Thermal Cycler (Applied Biosystems). Amplification of DNA sequences was achieved in cycles consisting of three main steps:

- Template DNA denaturation
- Annealing of primers
- Primer extension and polymerisation

Each step was carried out at a defined temperature for a specific period of time for 30-40 cycles. Denaturation of DNA was achieved by heating to 96-98°C for up to 30 seconds. The annealing temperature was dependent on the melting temperature (T_m) of the two custom primers, usually T_m°C -5°C for 30 seconds [where T_m°C = 4(G+C) +2(A+T)]. All reaction samples were heated to an extension temperature of 72°C (15 seconds per 1000 bases amplified for Phusion Flash Master Mix and 15 seconds per 100 base pairs for Bio Mix Red master mix). All samples were cooled to 4°C at the end of the cycles. Samples were then loaded directly onto an agarose gel for analysis (2.4.2) if BioMix Red master mix was used or purified using a kit (2.5.2) for subsequent cloning (2.7). Negative control reactions contained all components but no template DNA. A typical 50 µl sample for amplification by PCR would contain:

- 25 µl Phusion Flash master mix (x2) / BioMix Red master mix (x2) (contains necessary reagents, including $MgCl_2$)
- 2 µl template DNA (1-5 ng/µl)
- 2 µl forward primer (0.1-1µM)
- 2 µl reverse primer (0.1-1µM)
- 19 µl DNase, RNase-free UHQ dH_20

2.6.4 Sequencing

All DNA sequencing was completed by Source Bioscience (Rochdale, UK) who offered a Sanger sequencing service. This method works by denaturing DNA into single strands and a DNA polymerase binds to the primer to synthesise a new strand of DNA using

free nucleotides, including nucleotides that are fluorescently labelled. These fluorescently labelled nucleotides are read by a laser during capillary electrophoresis and the original DNA sequence is decoded. Sample requirements were as follows: 1ng/µl per 100 bp for products amplified by PCR, 100 ng/µl for plasmids and 3.2pmol/µl for primers. All samples sent for sequencing were purified using purification kits (2.5.1 and 2.5.2). Source Bioscience provided their own stock primers for sequencing recombinant DNA inserts in common plasmid vectors such as pRSET-A and pGEX-4T-1 (Table 2.2).

2.7 MANIPULATION OF NUCLEIC ACIDS

2.7.1 Restriction Digestion

Enzymes and their recommended/provided reaction buffers (mostly supplied as 10x concentrates) were stored at -20°C. If possible, High Fidelity (HF) enzymes (New England Biolabs) were used as these enzymes exhibit reduced star activity compared to non-HF enzymes. Digests contained 1-2 µg of DNA, 5-10 units of enzyme (New England Biolabs or Promega) per µg DNA, the recommended restriction digest buffer to a final concentration of 1x, and 1x BSA (if required) in total reaction volumes of 25-50 µl. If required, reactions were diluted in DNase and RNase-free UHQ H_20 (2.1.1). Reactions were incubated at 37°C (unless otherwise stated by the manufacturer) for 2-3 hours. If digests were used for subsequent cloning, reactions were purified by agarose gel electrophoresis (2.4.2) using a purification kit (2.5.2).

2.7.2 Dephosphorylation

Linearised vector DNA was dephosphorylated prior to cloning to prevent the plasmids from recircularising during ligation. The dephosphorylation reaction contained to up 2 µg purified digested DNA (2.7.1), up to 5 units of alkaline phosphatase (Antarctic Phosphatase (New England Biolabs) or Alkaline Phosphatase; Calf-Intestinal (Promega)), 1x recommended/supplied reaction buffer and 1x BSA (if required) in a total reaction volume of up to 100 µl. Reactions were diluted in DNase and RNase-free UHQ H_20 (2.1.1). Reactions were incubated at 37°C for up to 60 minutes and then 70°C for 15 minutes (New England Biolabs) for deactivation. The 37°C incubation step causes the ends of the DNA duplex to separate slightly allowing the enzyme access to the 5' terminal phosphate groups. For alkaline phosphatases that were unable to be

inactivated by heat (Promega), purification was conducted using a purification kit (2.5.2) before proceeding with ligation steps.

2.7.3 Ligation

Inserts generated by PCR (2.6) and plasmid vectors were digested using restriction endonucleases and dephosphorylated (if required) to facilitate their ligation to the vector. Rapid ligations were also performed using T4 DNA ligase (Promega). T4 DNA ligase facilitates the joining of two separate strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides. The 10µl reaction consisted of 10-100 ng dephosphorylated vector (2.7.2), a 3 fold molar excess of insert DNA, 1 unit of T4 DNA ligase and 1x Rapid Ligation Buffer (Promega). Rapid ligation reactions were incubated at room temperature for 15 minutes or 4°C overnight. Ligated plasmids were then transformed into competent *E.coli* cells (2.3.3).

2.8 RECOMBINANT PROTEIN EXPRESSION IN *E. COLI*

2.8.1 The pRSET-A expression vector

The pRSET-A/B/C (Invitrogen/Thermo Fisher Scientific) expression vectors are designed for high-level prokaryotic protein expression and purification and are under control of the T7 promoter. Expressed recombinant proteins are fused to a 7 kDa N-terminal polyhistidine tag and Xpress epitope. The six-histidine residues allow binding of the recombinant protein to a nickel binding domain for purification during immobilised metal affinity chromatography. Recombinant proteins expressed using pRSET-A/B/C expression vectors also contain an enterokinase cleavage recognition site between the fusion tag and the recombinant protein to allow removal of the N-terminal fusion tag from the recombinant protein.

2.8.2 The pGEX-4T-1/6P-1 expression vectors

Recombinant proteins using the GEX-4T1/6P-1 expression vectors are under control of the *tac* promoter and are fused to a 26 kDa soluble N-terminal glutathione s-transferase (GST) tag. This GST tag allows binding of the recombinant protein to a matrix such as Sepharose during affinity chromatography. Recombinant protein expressed using the pGEX expression vectors also contain a cleavage site between the N-terminal GST tag and recombinant protein to allow for the removal of GST. Cleavage sites in vectors

pGEX-1 to pGEX-4, pGEX-5 and pGEX-6 are recognised by thrombin, Factor Xa and Prescission Protease, respectively.

2.8.3 Cloning into the expression vectors

All insert fragments amplified by PCR (2.6) were purified (2.5), digested (2.7.1) and ligated (2.7.3) into pRSET-A or pGEX-4T-1/6P-1 expression vectors containing multiple cloning sites (MSCs). All clones were screened by sequencing (2.6.4) before transformation into competent *E. coli* cells (2.3.3) for protein expression.

2.8.4 Growth of E. coli and expression of recombinant protein

A single colony from a working stock (2.3.1) of *E. coli* on LB agar (2.2.2) was used to inoculate an overnight starter culture of 10 ml LB medium (2.2.1) with the relevant antibiotics (if required). A 2.5 ml aliquot of the starter culture was used to inoculate 250 ml SOB medium (2.2.4) and was incubated at 37°C on a 200 rpm shaker until OD_{600} absorbance value reached 0.4-0.6 on a spectrophotometer. A 1 ml aliquot of *E. coli* cells were collected as the time zero sample prior to induction using isopropyl β-D-1 thiogalactopyranoside (IPTG). 1 mM IPTG (Fisher Scientific) was added to the 250 ml culture and cells were incubated at 37°C for 2-4 hours. During pilot expression studies, hourly 1 ml samples were taken to determine the optimal induction time for the expression of the target protein. Otherwise, 1 ml samples were taken at the end of the induction period. Cells were pelleted by centrifugation at 4°C for 20 minutes at 7000 x *g*. Supernatants were discarded and cell pellets were frozen at -20°C. 1 ml sample aliquots of cells were pelleted by centrifugation at room temperature for 1 minute at 14,000 rpm. Supernatants were discarded and pellets were resuspended in 200 µl 1x PBS and frozen at -20°C. Sample aliquots of cells were analysed by SDS-PAGE (2.10) and analysed to determine optimal induction time for the target protein.

2.8.5 Lysis of E. coli cells

2.8.5.1 Sonication

Sonication used high frequency sound energy to agitate samples and disrupt cell membranes, resulting in cell lysis. To analyse 1 ml cell culture samples from pilot expression studies, cells were lysed by sonication using a MSE Sanyo Soniprep 150. Samples were pulsed for 10 seconds at 3-4 amplitude microns with 5 second intervals and repeated for 2-3 cycles.

2.8.5.2 BugBuster Master Mix

Lysis of *E. coli* cells from 250 ml batch cultures (2.8.4) was achieved using BugBuster Master Mix (Novagen, Merck Millipore) using the manufacturer's protocol. BugBuster Master Mix contains Benzonase Nuclease and rLysozyme in a non-denaturing Tris-based buffer and this allows the lysis of large cell culture batches without heating or sonication. Benzonase Nuclease reduces viscosity caused by chromosomal DNA and rLysozyme hydrolyzes N-acetylmuramide linkages in the peptidoglycan layer of the cell wall. However, this is unnecessary when proteins are expressed from host strains containing the pLysS plasmid, e.g. BL21 (DE3) pLysS, as these strains express T7 lysozyme. Cell pellets containing induced target proteins (2.8.4) were frozen and thawed on ice. 10 ml ice-cold BugBuster Master Mix and one cOmplete Protease Inhibitor Cocktail tablet (Roche Applied Science) were added to the cell pellets and vortexed until the cells were completely resuspended. The cells were kept on ice for 20 minutes on a rocker with gentle agitation. Lysed cells were pelleted by centrifugation at 16,000 x *g* for 20 minutes at 4°C. Supernatants were kept for analysis of soluble target proteins by SDS-PAGE (2.10). Lysed pellets containing insoluble proteins inside inclusion bodies were washed by suspending in 5 ml 10% (v/v) BugBuster Master Mix diluted in 1x PBS and centrifuged at 5000 x *g* for 15 minutes at 4°C. This step was repeated four times to release insoluble proteins from inclusion bodies. All samples were kept for analysis by SDS-PAGE (2.10) and stored at 4°C until purification on the same day (2.9).

2.9 PURIFICATION OF RECOMBINANT PROTEINS

2.9.1 ProBond Resin

Probond Resin (Novex, Life Technologies) precharged with Ni^{2+} ions possess a high affinity for histidine residues expressed as fusion tags with recombinant proteins using pRSET-A/B/C expression vectors (2.8.1). This allows for the purification of recombinant target proteins bound to the resin using imidazole by affinity chromatography. It is provided as 50% slurry stored in 20% ethanol. To remove the ethanol, the slurry is washed twice in UHQ dH_20.

2.9.2 Glutathione sepharose 4B

The structure of glutathione bound to Sepharose 4B Beads (GE Healthcare) is complementary to the glutathione s-transferase (GST) binding site of recombinant proteins expressed with a GST tag using pGEX expression vectors (2.8.2). This allows for the purification of recombinant target proteins using reduced glutathione by affinity chromatography. The glutathione is coupled to 4% agarose and is stored in 20% ethanol. To remove the ethanol, the slurry is washed twice in UHQ dH_20.

2.9.3 Dialysis of proteins

Purified aliquots of proteins were pooled together for dialysis. Fisherbrand regenerated cellulose dialysis tubing (Fisher Scientific) was autoclaved in 1x PBS (2.1.2), tied and clamped before being carefully loaded with protein. The tubing was tied off and clamped and was placed in 1 litre of dialysis buffer per 1 ml of protein. Tubing and buffers were kept at 4°C. Dialysis buffer was changed after 3 and 6 hours before leaving overnight at 4°C. Proteins were carefully removed and stored at -20°C in 200 µl aliquots.

2.10 ANALYSIS OF PROTEIN

2.10.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) separates linear polypeptide SDS-complexes by electrophoretic molecular sieving in polyacrylamide gels. Glass plates (BioRad) and spacers (1.5 mm) used to form the gel mould were cleaned and degreased with 70% (v/v) Industrial Methylated Spirit (IMS). All buffers and solutions were prepared using dH_20. Materials and stock solutions used for SDS-PAGE are shown in Table 2.3. SDS-PAGE was performed with 12.5% separating gels, unless otherwise stated, and prepared as shown in Table 2.4. Polymerisation of acrylamide to form gels was accelerated by free radicals from N, N, N'-N'-tetramethylethylenediamine (TEMED) generated by the action of the initiator ammonium persulphate (APS). The separating gel was first cast and overlaid with 100% isopropanol to ensure the gel surface was flat and air was excluded. Once the separating gel had set (approximately 30 minutes), the isopropanol was removed by rinsing the surface several times with dH_20. The stacking gel was then carefully poured onto the top of the separating gel. A plastic well comb was added and the gel was allowed to set

(approximately 20 minutes). For each SDS-PAGE run, fresh electrode buffer was prepared. Well combs were removed and the gels with or without a buffer dam were assembled in the electrophoresis tank (BioRad MiniPROTEAN Tetra System). Protein samples were prepared as described in 2.10.1.1. Electrophoresis was carried out at 200 volts for approximately 60 minutes until there was sufficient separation between bands in the protein marker (2.10.2).

2.10.2 Analysis of native and denatured proteins

Proteins that are denatured by SDS, β-mercaptoethanol (BME) and heat destroy secondary and tertiary structures and disulphide bonds. This allowed for the observation of monomeric proteins with an approximate equal charge to mass ratio separated by molecular weight. For denatured samples, 10 µl aliquots of proteins or whole cultures of cells were treated in SDS buffer (1.25% BME, 2% SDS, 0.01% bromophenol blue, 10% glycerol, 62.5 mM Tris-HCl [pH 6.8]) and boiled for 5 minutes at 100°C. To analyse proteins under native conditions, buffer treatments were optimised and tailored to each protein. Generally, proteins were treated in 0.01% bromophenol blue, 10% glycerol, and 62.5 mM Tris-HCl [pH 6.8], were not heat-treated and were analysed by SDS-PAGE using 12.5% SDS acrylamide gels.

2.10.3 Protein markers

Molecular weights of proteins analysed by SDS-PAGE were estimated by comparing electrophoretic mobility with proteins of known molecular weight. 5-10 µl PageRuler Prestained Protein Ladder (ThermoFisher Scientific) was loaded directly into the wells of polyacrylamide gels (no loading buffer or heat treatment was required). This protein marker contains ten proteins of known molecular weight ranging from 10-180 kDa which are visible on the polyacrylamide gel prior to gel staining (2.10.4) and can be transferred to a PVDF membrane (2.10.7). The ladder was stored at -20°C.

2.10.4 Protein staining

A glass or plastic dish used for the staining procedure was cleaned with 100% (v/v) IMS. The gel was placed in staining solution (0.1% (w/v) Brilliant Blue (Fisher Scientific), 10% (v/v) acetic acid, 20% (v/v) isopropanol) for 1 hour or until bands were clearly visible. Excess staining solution was removed by repeated washes in destaining solution (10% (v/v) acetic acid, 20% (v/v) isopropanol) until the background was clear.

Table 2.3 Materials and stock solutions used for SDS-PAGE

Solution	Contents
Bis-Acrylamide solution 37.5:1 (Fisher Scientific)	40% stock solution – stored at 4°C
SDS stock solution (Fisher Scientific)	10% (w/v) in UHQ dH_20
Separating buffer	1.5 M Tris-HCl [pH 8.8]
Stacking buffer	0.5 M Tris-HCl [pH 6.8]
N, N, N'-N'-tetramethylenediamine	TEMED stock (BioRad/Fisher Scientific)
Ammonium persulphate solution (Fisher Scientific)	10% (w/v) – stored at -20°C
Electrode buffer	20 mM Tris, 192 mM glycine, 0.1% (w/v) SDS – freshly prepared in dH_20 from 10x stock
Denaturing sample buffer (x4 stock)	250 mM Tris-HCl [pH 6.8], 8% (w/v) SDS, 40% (v/v) glycerol, 5% (v/v) BME, 40 µg (0.004%) bromophenol blue
Native sample buffer (x4 stock)	250 mM Tris-HCl [pH 6.8], 40% (v/v) glycerol, 40 µg (0.004%) bromophenol blue

Table 2.4 Components of one SDS-PAGE gel

Solution	Separating gel (10%)	Separating gel (12.5%)	Stacking gel (5.3%)
Bis-Acrylamide 37.5:1	1.25 ml	1.5 ml	225 µl
Separating buffer	1.9 ml	1.9 ml	N/A
Stacking buffer	N/A	N/A	1 ml
UHQ dH_20	1.75 ml	1.5 ml	675 µl
10% SDS	50 µl	50 µl	20 µl
APS (10%)	20 µl	20 µl	10 µl
TEMED*	5 µl	5 µl	2 µl

*added immediately before casting the gel

2.10.5 Gel drying

Polyacrylamide gels that had undergone staining and destaining (2.10.3) were placed in gel drying solution (5% (v/v) glycerol, 20% (v/v) methanol) overnight and dried between two sheets of gel drying film using a gel drying kit (Promega).

2.10.6 Protein quantification

Pierce 660 nm protein assay reagent (ThermoFisher Scientific) was used to quantify proteins. This assay is based on the Bradford assay and is not affected by purified proteins containing trace amounts of reduced glutathione or imidazole. 1.5 ml of reagent was added to 100 µl protein and incubated at room temperature for 5 minutes. Samples were read at 660 nm on a spectrophotometer. A blank sample was prepared using the protein storage buffer. Standard curves were produced using known concentrations of bovine serum albumin (BSA) (ThermoFisher Scientific) ranging between 5-2000 µg/ml. All samples and standards were analysed by SDS-PAGE (2.10.1) for confirmation.

2.10.7 Electrophoretic protein transfer

Protein samples were analysed by SDS-PAGE with a protein marker (2.10.1-2.10.3) 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 for 7.5 minutes at 25 volts. PVDF membranes were prepared by soaking in 100% methanol and rinsing in Fast Semi-Dry Buffer. Gels were prepared by rinsing in UHQ dH_20 and Fast Semi-Dry Buffer.

2.10.8 Detection of proteins by Western blot

Western blotting, also called immunoblotting, allows the detection of a target protein by applying an antibody directly to a membrane. Following electrophoretic transfer of proteins (2.10.7), membranes were treated in blocking buffer (10% skimmed milk (Marvel) in 1x PBS with 0.1% Tween-20 (PBS-T)) for 1 hour at room temperature. This step blocks all free binding sites to avoid non-specific binding of antibodies. The membrane was incubated with primary antibody at the appropriate dilution in PBS-T with 1% milk for 1 hour at room temperature. Unbound antibody was removed by three x 5 minute washes in PBS-T. Secondary antibody was added at the appropriate dilution

in PBS-T with 1% milk for 1 hour at room temperature and washed as described. The detection method was dependent on the secondary antibody conjugate.

2.10.8.1 Antibodies

Antibodies used for the detection of proteins by western blot included monoclonal mouse anti-his IgG diluted to 1:500 (Invitrogen), monoclonal mouse anti-GST IgG at 1:500-1:1000 (Invitrogen), polyclonal rabbit anti-PGP3 IgG at 1:1000 (derived from *C. trachomatis* pLGV440 (Gillian S. Wills, Imperial College London)), and polyclonal rabbit anti-PGP3 IgG at 1:2000 (derived from *C. trachomatis* pSW2 (produced at University of Southampton (2.12)). Primary antibodies also included human serum samples diluted at 1:100. If human serum samples produced high levels of false-positive background, samples were diluted to 1:200. All primary antibodies were detected using goat anti-rabbit (Sigma-Aldrich), mouse (BioRad) or human IgG-HRP (BioRad) conjugates diluted to 1:2000 and visualised using the ECL system (2.10.8.3). If high false-positive backgrounds were produced using human serum samples, antibodies were detected using goat anti-rabbit (Sigma-Aldrich), mouse (Sigma-Aldrich) or human IgG-AP (BioRad) conjugates diluted to 1:2000 and visualised using the colorimetric system (2.10.8.2). Unless otherwise stated, all antibodies were stored at -20°C.

2.10.8.2 BCIP-NBT

Primary antibodies detected by secondary antibodies conjugated to alkaline phosphatase (AP) were visualised by directly coating the membrane in 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) solution [100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5 containing 160 µg/ml BCIP and 400 µg/ml NBT] on a shaker until bands were visible (maximum 3 hours). To stop the reaction, membranes were rinsed several times in UHQ dH_20. Membranes were dried and stored at room temperature.

2.10.8.3 Enhanced Chemiluminescence (ECL) System

Primary antibodies detected by secondary antibodies conjugated to horseradish peroxidase (HRP) were visualised using Pierce enhanced chemiluminescence (ECL) system western blotting substrate (ThermoFisher Scientific). 1 ml of reagents 1 and 2 (supplied) were added to the membrane and incubated at room temperature for 1 minute. The membrane was exposed to X-ray film (CL-XPosure Film, ThermoFisher

Scientific) in a development box, for 5 seconds to 10 minutes in a dark room and placed in developing solution (Carestream Kodak autoradiography GBX developer (Sigma-Aldrich)) until bands were visible. The film was washed in water and placed in fixing solution for 2 minutes (Carestream Kodak processing chemicals for autoradiography (Sigma-Aldrich)). The photographic film was washed under water and left to dry.

2.11 INDIRECT ENZYME-LINKED IMMUNOSORBENT ASSAY

Indirect ELISAs were used to detect the presence of a specific antibody within a serum sample. Protocols were optimised for each capture antigen and detection antibody. Capture antigens were diluted to the appropriate concentration in 50 mM carbonate-bicarbonate buffer pH 9.6 (3.7 g $NaHCO_3$ and 0.64 g Na_2CO_3 in 1 litre of UHQ dH_20, sterile filtered (2.1.2) and stored at 4°C). Medisorp flat-bottom 96-well non-sterile plates (Nunc, ThermoFisher Scientific) were coated in antigen (100 µl/well), covered in cling film and incubated statically for 2 hours at 37°C. Plates were manually washed three times in 0.1% PBS-Tween 20 (PBS-T) by filling the wells and leaving each wash to incubate for at least 1 minute. Unless otherwise stated, unbound binding sites were 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 and primary antibodies were added (50 µl/well) at the appropriate dilution for 1 hour at 37°C. Plates were washed six times in PBS-T and incubated with secondary antibody (100 µl/well) at the appropriate dilution for 1 hour at 37°C. Unless otherwise stated, all 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 immediately read at $O.D_{450}$ using a BioRad iMark microplate absorbance reader.

2.11.1 Antibodies

Positive control antibodies used for the detection of proteins by ELISA included monoclonal mouse anti-his IgG diluted to 1:500 (Invitrogen), monoclonal mouse anti-GST IgG at 1:500-1:10,000 (Invitrogen), polyclonal rabbit anti-PGP3 IgG at 1:10,000 (derived from *C. trachomatis* pLGV440 (Gillian S. Wills, Imperial College London)), and polyclonal rabbit anti-PGP3 IgG at 1:20,000-100,000 (derived from *C. trachomatis* pSW2 (produced at University of Southampton (3.2.3.2)). Primary antibodies also

included human serum samples diluted at 1:100 or mouse sera diluted 1:50. All primary antibodies were detected using goat anti-rabbit (Sigma-Aldrich), mouse (BioRad) or human IgG-HRP (BioRad) conjugates diluted to 1:8000.

2.12 ETHICS

The work with human sera is covered by two NHS research ethics committee approved studies submitted through IRAS:

1) Chlamydial antibodies in infection (REC no: 16/NW/0346).

2) Seroepidemiological surveillance of the national immunisation programme of England and Wales (REC no: 05/Q0505/45).

Both these projects are registered with Ethics and Research Governance Online (ERGO) University of Southampton according to the University regulations.

2.13 DATA ANALYSIS AND SOFTWARE PACKAGES

Unless otherwise stated, statistical analyses were conducted using GraphPad Prism version 6.0 (GraphPad Software Inc., USA) to calculate one or two-way ANOVA and two-tailed t tests stating the mean, standard deviations and standard errors. GraphPad Prism version 6.0 was also used to calculate sensitivities and specificities of assays using Receiver Operating Characteristic (ROC) curve analyses and intra- and interassay variation of assays. Results were considered to be statistically significant if $p = <0.05$. DNA and amino acid sequences were viewed and aligned using SeqBuilder and MegAlign programs from DNAStar Lasergene version 8.0 and 14.0 software packages (Lasergene, Madison, WI, USA). Sequencing data was analysed using FinchTV version 1.4 chromatogram software (Geospiza Inc., WA, USA). Serum details and background-corrected OD_{450} results obtained from ELISAs were organised and calculated using Microsoft Excel from Microsoft Office 2010. Flow diagrams were created using EDraw Max version 7 (2013).

2.14 STATISTICAL ANALYSIS

2.14.1 One/ two-way Analysis of Variance (ANOVA) and multiple comparison tests

One-way ANOVA is a parametric test used to compare the differences of two or more sets of unrelated measurements e.g. to test whether multiple experimentally treated

groups are different from the controlled. Two-way ANOVA is similar to one-way ANOVA but it also investigates the effects of two factors at once. GraphPad Prism version 6.0 was used to calculate one and two-way ANOVA. Tukey's test was used as a post-test for comparison of multiple groups with each other. The Dunnett test is a multiple comparison test similar to the Tukey test but compares multiple groups with a control group.

2.14.2 Two-tailed t-test

The two-tailed t-test, also known as the independent sample t-test, is a parametric test used to compare the means of two independent (unpaired) sets of measurements e.g. to test whether an experimentally treated group is different from the controlled.

2.14.3 Sensitivity and specificity

Sensitivity and specificity are terms used to evaluate the accuracy of an assay. The sensitivity of an assay refers to e.g. the ability of a test to correctly identify the presence of a particular antibody in patient serum ('true positives'). An assay with a 100% sensitivity score will correctly detect the presence of the antibody in all patient serum. Likewise, the specificity of an assay refers to the ability of a test to correctly confirm the absence of a particular antibody in patient serum ('true negatives'). An assay with a 100% specificity score will confirm the absence of the antibody in all patient serum.

2.14.4 Receiver Operating Characteristic (ROC)

A receiver operator curve, also known as receiver operating characteristic (ROC), is a test used to evaluate the accuracy of an assay. The true positive fractions (sensitivity) are plotted against the false positive fractions (specificity) for various cut-off points . An assay with a perfect discrimination (100% sensitivity and 100% specificity) will have an ROC area value of 1. This value decreases with increased inaccuracy of the assay. A value of 0.5 indicates a useless assay.

2.14.5 Chi-square for Associations and Fisher's Exact

The chi-square (X^2) test for associations is a test used to test for an association or difference between the test frequencies of two or more groups. It is used when there is no expected frequency, whereas the chi-square test for differences would be used to be test for differences when there is an expected frequency. The Fisher's Exact test is

similar to the Chi-Square test for associations. It produces an accurate p value whereas chi-square tests only produce an approximate p value and it is used when group sample sizes are small in number. Chi-square tests are only calculated using raw numbers of observations (e.g. cases of positives and negatives, not percentages).

CHAPTER THREE: EXPRESSION, PURIFICATION AND DETECTION OF RECOMBINANT PROTEINS

3.1 INTRODUCTION

As previously discussed, PGP3 is encoded by coding sequence 5 (CDS5) of the *C. trachomatis* plasmid and the 264 amino acid protein products polymerise to form an 84 kDa homotrimer (158). PGP3 has been shown to play a role in virulence as it has recently been reported that PGP3 binds to and neutralises LL-37, an antimicrobial peptide secreted by epithelial cells and leukocytes (182). Furthermore, 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. These properties have made PGP3 an ideal antigen for detecting anti-*C. trachomatis* antibodies for use in ELISA formats, as described previously (75, 118, 123, 160, 163, 167, 168). These ELISAs incorporated recombinant PGP3 to detect anti-PGP3 antibodies in patient serum. However, the methods of recombinant PGP3 purification and the PGP3 serovar derivation vary between all studies.

Previous studies using PGP3 in an ELISA to detect anti-PGP3 antibodies used recombinant PGP3 derived from various serovars of *C. trachomatis*, including L1 (118) and a urogenital strain, D (75, 123, 160, 163, 167, 168). PGP3 derived from *C. trachomatis* serovar D (GenBank: NC_001372) shares 100% amino acid identity to PGP3 derived from E (EMBL: FM865439). The CDS5 DNA sequence is highly conserved amongst urogenital isolates (126). However, PGP3 from urogenital serovar E and LGV serovar L1 vary by nine amino acids with different hydrophobic/hydrophilic characteristics (Figure 3.1). These variations are reflected in the different isoelectric points (pI) of PGP3 between serovars E and L1 (4.34 and 4.57, respectively) which could account for potential structural differences which may the overall sensitivity of an ELISA. Despite this, Wills *et al.* (2009) (118) developed an ELISA to detect prior exposure to *C. trachomatis* in patient sera based on PGP3 derived from the *C. trachomatis* pLGV440 plasmid.

In addition to differences in the serovar derivation of recombinant PGP3 used in ELISAs, there is also variation between the recombinant protein purification methods.

A summary if the purification methods and PGP3 serovar derivation used by these ELISAs can be seen in Tables 1.4 and 1.5. The expression and purification of recombinant PGP3 using denaturing purification methods have been described (118, 160, 174). These methods may alter the native trimeric conformation of PGP3. As previous reports have demonstrated that the human antibody recognition of PGP3 is dependent on its native trimeric conformation (124, 125), the purification of recombinant PGP3 using native purification methods is essential. Methods for the native purification of recombinant PGP3 have also been described (75, 123, 163, 167, 168). However, there is significant variation within these methods, including the recombinant protein expression vector used and the resulting N- or C-terminal polyhistidine or glutathione s-transferase tag (if applicable), although the reasons for either retaining (123, 160, 167, 168) or subsequently cleaving (118) these fusion tags are not made clear in these studies. Collectively, the differences in recombinant protein expression and purification methods, as well as PGP3 serovar derivation, may alter the conformation of PGP3 and affect the overall sensitivity and specificity of the ELISA. This chapter describes the expression and native purification of recombinant PGP3 derived from *C. trachomatis* plasmids pSW2 and pLGV440 (serovars E and L1, respectively).

3.1.1 Aims

- Develop a simple, reproducible protocol incorporating native purification using affinity chromatography to express and purify recombinant PGP3 to use as a capture antigen.

3.1.2 Objectives

- Amplify and clone CDS5 derived from *C. trachomatis* plasmids pSW2 and pLGV440 into expression vectors pRSET-A and pGEX-4T-1/6P-1.
- Express and purify recombinant PGP3 from pSW2 and pLGV440 with an N-terminal polyhistidine or glutathione s-transferase (GST) tag using affinity chromatography.
    - Express and purify mouse norovirus 3C protease with an N-terminal polyhistidine tag and GST using affinity chromatography to use as negative control antigens to assess potential cross-reactivity from human sera to the his- and GST tags in an ELISA or Western blot.

- Express and purify recombinant PGP3 from pSW2 and pLGV440 enzymatically cleaved from the N-terminal GST tag.
- Use cleaved PGP3 from pSW2 to immunise a rabbit to produce rabbit polyclonal anti-PGP3 positive control antibody.
- Use mouse monoclonal anti-his/GST and rabbit polyclonal anti-PGP3 antibodies in a Western blot to detect recombinant monomeric and trimeric PGP3 and PGP3 expressed in *C. trachomatis* and *C. muridarum* elementary bodies.

Figure 3.1 Amino acid alignment of PGP3 sequences expressed from coding sequence five (CDS5) from the *C. trachomatis* serovars E (pSW2) and L1 (pLGV440).

```
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
```

3.2 METHODS AND MATERIALS

Methods and materials relevant to this chapter are described here. For more detailed methods and materials, see chapter two.

3.2.1 Cloning of CDS5 (PGP3) in E. coli expression vectors

C. trachomatis plasmid templates pCTL12A (serovar L1) and pSP73-SW2 (serovar E) (Table 3.1) were purified (2.5.1) following overnight culture in LB (2.3.1). Purified templates were used to amplify CDS5 by PCR (2.6) using the primers in Table 3.2 and amplification conditions in Table 3.3. The endonuclease restriction sites, BamHI and XhoI, were incorporated into the forward and reverse primers, respectively.

Amplified 795 bp inserts encoding PGP3 were analysed by agarose gel electrophoresis (2.4.2), gel extracted using a purification kit (2.5.2) and digested as described (2.7.1). Expression vectors pRSET-A (2.8.1) and pGEX-4T-1/6P-1 (2.8.2) were cultured overnight (2.3.1), purified (2.5.1), digested (2.7.1) with BamHI and XhoI and dephosphorylated (2.7.2) as described.

Amplified CDS5 inserts and expression vectors were ligated as described (2.7.3) and transformed into competent DH5 alpha *E. coli* (2.3.3). After transformation, this strain of *E. coli* will contain the expression plasmids and be resistant to ampicillin, allowing for selection and screening of recombinant clones. Transformed *E. coli* were sub-cultured onto LB Amp plates (2.2.6), grown overnight in LB (2.3.1) and purified by STET (2.5.3). Sizes of the recombinant plasmid clones were analysed against empty/uncloned vectors by agarose gel electrophoresis (2.4.2). Constructs of the expected sizes containing the CDS5 insert (pRSET-A-CDS5: 3.5 kb; pGEX-4T-1/6P-1-CDS5: 4.7 kb) were cultured in LB overnight with ampicillin (2.3.1), purified (2.5.1) and sent for sequencing (2.6.4) to confirm the presence and correct DNA sequence of the insert. Sequencing data was analysed using FinchTV and sequences were analysed against CDS5 sequences using MegAlign from DNAStar Lasergene (2.13). Recombinant plasmids containing CDS5 were transformed into competent BL21 (DE3) pLysS *E. coli* for protein expression. Expression of recombinant protein in this strain is under the control of a T7 promoter. This is recognised by T7 RNA polymerase which is induced by the addition of IPTG. Construction of the pRSET-A and pGEX-4T-1/6P-1 plasmid clones containing the CDS5 sequence is depicted in Figures 3.2 and 3.3.

Table 3.1. Plasmid template list

Plasmid Name	Origin/ Source	Host	Resistance	Information	Reference
pSP73-SW2	pSW2	*E. coli* DH5α	Ampicillin	cc 1,118 (2007)	Made by Yibing Wang
pCTL12A	pLGV440	*E. coli* JM83	Ampicillin	cc 19 (1985/2011) pLGV440 excised from pCTL1	Hatt *et al.* (1988) (183)

Table 3.2. Primer list

Primer Name	Target	Sequence (5'-3')	Size (bp)	Sites
CDS5_ Forward	CDS5 on *C. trachomatis* plasmid (not serovar-specific)	gcagcaGGATCC atgggaaattctggtttttatttgt	37	BamHI
CDS5_ Reverse	CDS5 on *C. trachomatis* plasmid (not serovar-specific)	gcagcaCTCGAG attgtttaagcgtttgtttgagg	35	XhoI

Table 3.3. PCR conditions for the amplification of CDS5 on *C. trachomatis* plasmids

	Time (secs/min)	Temp (°C)	
Stage 1	10 secs	98	
Stage 2	1 secs	98	**35 cycles**
	5 secs	62	
	15 secs	72	
Stage 3	1 min	72	
Finish	Continuous	4	

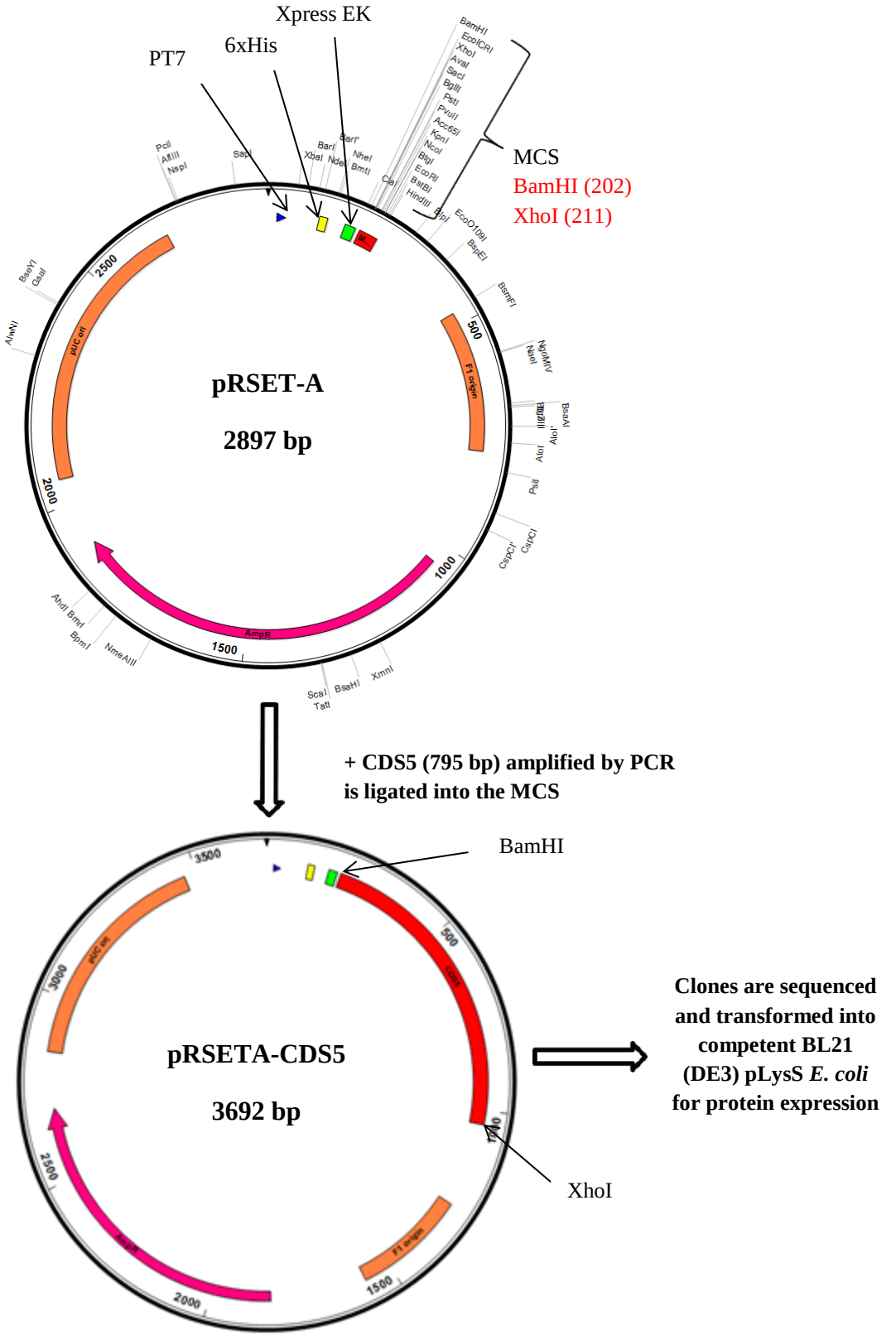


Figure 3.2. Cloning strategy for the generation of the pRSETA-CDS5 expression vectors. Coding sequence 5 from *C. trachomatis* plasmids pLGV440 and pSW2 was amplified by PCR and ligated into the pRSET-A expression vector using BamHI and XhoI restriction endonucleases. MCS = multiple cloning site

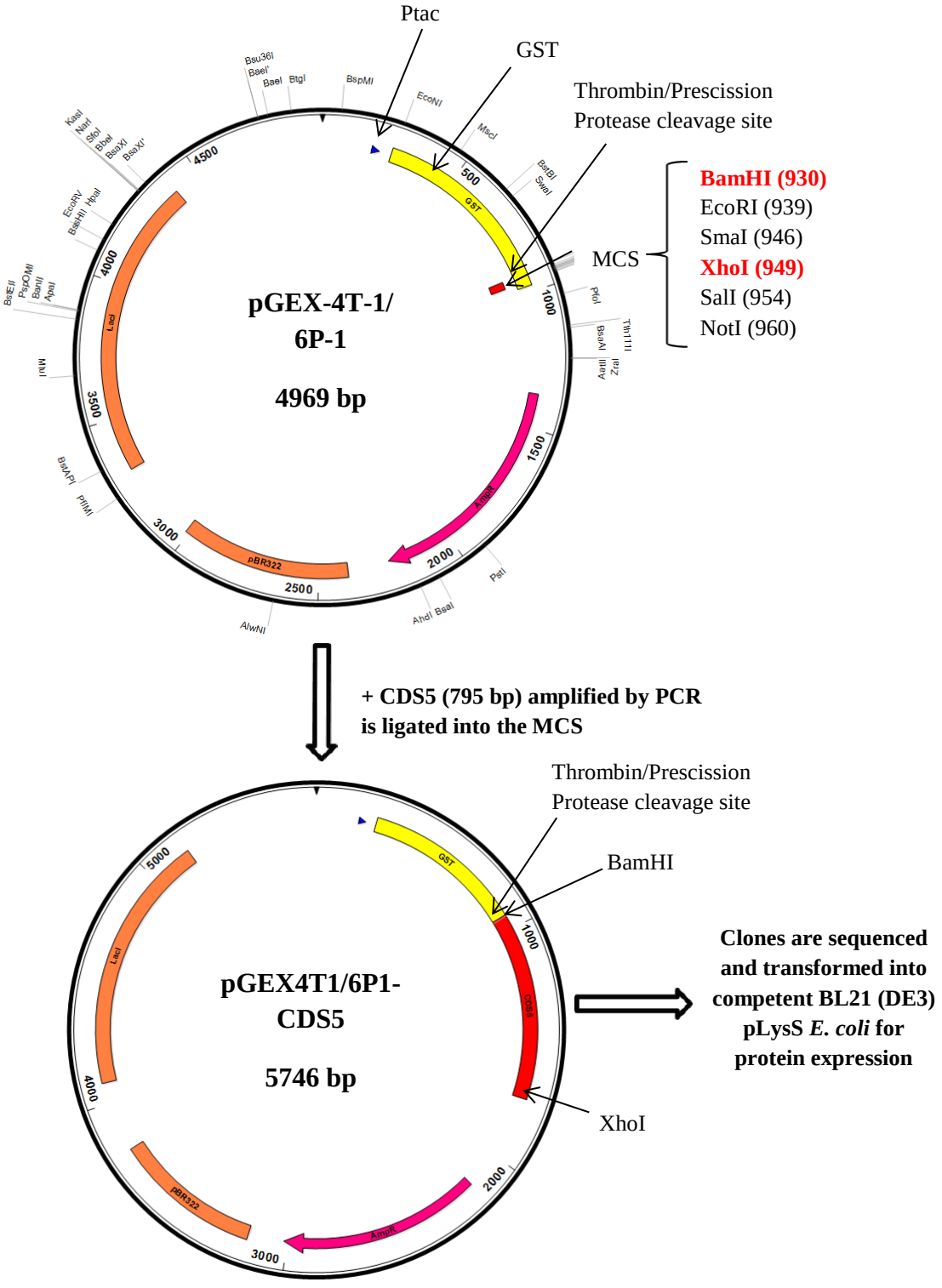


Figure 3.3. Cloning strategy for the generation of the pGEX4T1/6P1-CDS5 expression vectors. Coding sequence 5 from *C. trachomatis* plasmids pLGV440 and pSW2 was amplified by PCR and ligated into the pGEX-4T-1/6P-1 expression vectors using BamHI and XhoI restriction endonucleases. MCS = multiple cloning site

3.2.2 Recombinant protein expression and purification

All recombinant proteins expressed and purified using fusion tags had undergone pilot expressions to determine optimal induction times (2.8.4). All proteins had undergone multiple purification experiments to determine the optimal purification conditions. Proteins and reagents were kept on ice during purification and purified proteins were stored at -20°C in their elution buffers until dialysis steps. Unless otherwise stated, purified proteins were dialysed into PBS (2.9.3) and frozen at -20°C in 200 µl aliquots. Protein concentrations were estimated using SDS-PAGE (2.10) on 12.5% SDS gels using known concentrations of bovine serum albumin (BSA) and quantified using the Pierce 660 nm protein assay reagent (ThermoFisher Scientific) according to the manufacturer's protocol (2.10.6).

BL21 (DE3) pLysS *E. coli* cells transformed with pRSET-A (2.8.1) and pGEX-4T-1/6P-1 (2.8.2) recombinant clones were cultured overnight (2.3.1), grown in SOB medium (2.2.4) and induced with 1 mM IPTG (2.8.4) as described. Induced cells were pelleted, freeze-thawed and lysed using BugBuster Master Mix (2.8.5.2), as described. Expression and purification of recombinant proteins is depicted in figure 3.4.

3.2.2.1 Purification of recombinant his-PGP3 and his-3C proteins using Probond Resin

Probond Resin precharged with Ni^{2+} ions (2.9.1) possess a high affinity for histidine residues expressed as fusion tags with recombinant proteins using pRSET-A/B/C expression vectors (2.8.1). This allowed for the purification of target proteins using affinity chromatography under native conditions. For a 250 ml cell culture (2.8.4), 2 ml resin was prepared by washing in UHQ dH_20 twice to remove excess ethanol from the storage buffer. Lysed protein-containing fractions (2.8.5.2) were incubated with the resin for 1 hour at 4°C with buffer A (Table 3.4). Resin beads containing bound his-tagged protein were settled by incubation for 5-10 minutes or until there was a clear distinction between the beads and supernatant. The supernatant was removed and kept for analysis by SDS-PAGE (2.10). Beads were washed in 4 ml buffers B and C and supernatants removed as described. The beads were loaded onto a disposable plastic chromatography column and final excess wash buffers were removed. The beads were incubated with 5 ml elution buffer D for 1 minute and flow through containing purified

the target his-tagged protein was removed in 1 ml aliquots. All wash and elution aliquots were kept for analysis by SDS-PAGE on 12.5% gels (2.10).

3.2.2.2 Purification of recombinant glutathione s-transferase-PGP3 and GST proteins using Glutathione 4B Sepharose Beads

The structure of glutathione bound to Sepharose 4B Beads (GE Healthcare) is complementary to the glutathione s-transferase (GST) binding site (2.9.2). This allowed for the affinity purification of target proteins fused to a GST tag expressed using pGEX expression vectors (2.8.2). For a 250 ml cell culture (2.8.4), 650 µl bead slurry was prepared by washing in UHQ dH_20 twice to remove excess ethanol from the storage buffer. Lysed protein-containing supernatant fractions (2.8.5.2) were incubated with the resin for 1 hour at room temperature in 10 ml of buffer A (Table 3.5). Resin beads containing bound GST-tagged protein were settled by incubation for 5-10 minutes or until there was a clear distinction between the beads and supernatant. The supernatant was removed and kept for analysis by SDS-PAGE (2.10). Beads were loaded onto a disposable plastic chromatography column and excess flow through was collected. The beads were washed three times in 2 ml buffer A and the flow through was collected for analysis. Beads were incubated with 1 ml buffer B for 1 minute and flow through containing GST-tagged protein was removed. Buffer B was added four more times in 1 ml aliquots and the beads were resuspended in each aliquot before eluted protein was collected.

3.2.2.3 Purification of recombinant PGP3 cleaved from glutathione s-transferase tags using Prescission Protease

Lysed fractions of induced *E. coli* cells containing recombinant target protein were incubated with the glutathione sepharose resin as described (3.2.2.2). Beads containing bound protein were washed twice in 2 ml buffer A and three times in 2 ml buffer C. Target proteins still bound to glutathione sepharose beads were cleaved from the GST tag using 48 units of Prescission Protease (GE Healthcare) in 1 ml of cleavage buffer C. The column was closed at both ends using the provided removable caps and added to a spinning rotator for 4 hours at room temperature, then 4°C overnight. 4 ml of buffer C was added to the beads and cleaved target protein was removed and collected as flow

through in one aliquot. All wash and elution aliquots were kept for analysis by SDS-PAGE on 12.5% gels (2.10).

Table 3.4. Wash and elution buffers for purification of recombinant his-tagged proteins

Buffer	Buffer name/Function	Contents
A	Binding	10 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl [pH 7.9]
B	Wash	25 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl [pH 7.9]
C	Wash	50 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl [pH 7.9]
D	Elution	250 mM imidazole, 500 mM NaCl, 50 mM Tris-HCl [pH 7.9]

Table 3.5. Wash and elution buffers for purification of recombinant glutathione s-transferase-tagged proteins

Buffer	Buffer name/function	Contents
A	Wash	1x PBS
B	Elution	50 mM reduced glutathione, 50 mM Tris-HCl [pH 8]
C	Cleavage	50 mM Tris-HCl, 150 mM NaCl, 1 mM DTT, 1 mM EDTA [pH 7], up to 50 units Prescission Protease

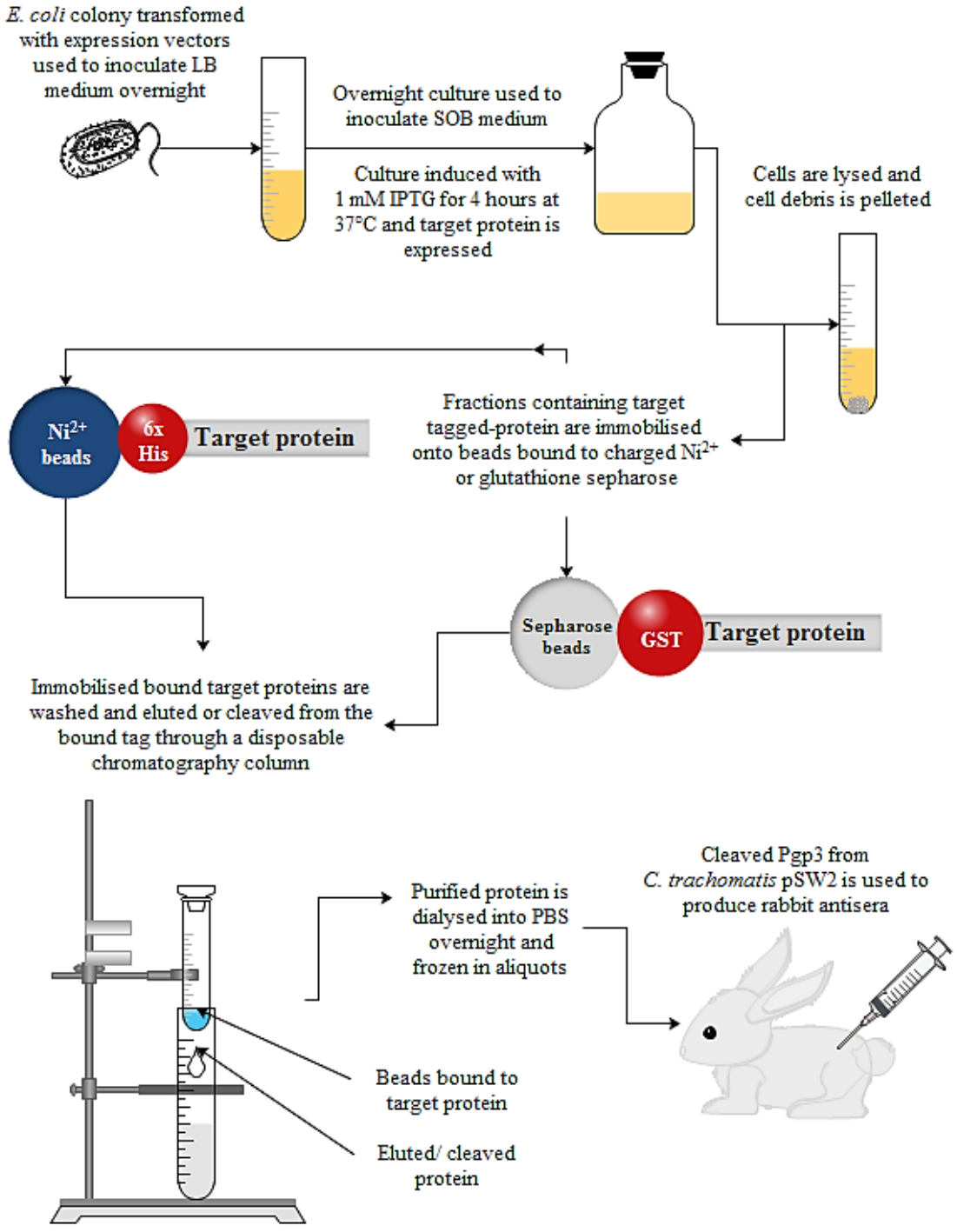


Figure 3.4. Strategy for the expression and purification of recombinant target proteins using affinity chromatography.

Only purified cleaved PGP3 from *C. trachomatis* pSW2 (serovar E) was used to produce rabbit polyclonal anti-PGP3 antibodies.

3.2.3 Production of polyclonal antibodies

3.2.3.1 Ethical statement

Procedures for the production of rabbit antisera to purified recombinant protein were carried out under Home Office project licence PPL30/3126 and Animals (Scientific Procedures) Act 1986.

3.2.3.2 Procedure

Recombinant protein was purified (3.2.2) and diluted to 50 µg aliquots in PBS. Freund's Complete Adjuvant (Sigma-Aldrich) was added to the antigen to an approximate 1:1 ratio (maximum of 1 ml) and was used to immunise one 3-month old female New Zealand white rabbit by intramuscular injection. Freund's Complete Adjuvant contains heat-inactivated and dried *Mycobacterium tuberculosis* in paraffin oil that induces a Th1-dominated inflammatory response at the site of injection. Three fortnightly immunisation boosters were followed using antigen preparations containing Freund's Incomplete Adjuvant (Sigma-Aldrich). The rabbit was weighed daily and physical health was monitored for signs of illness. Samples of sera were collected before the first and after the third immunisation to confirm the absence and presence of anti-PGP3 antibodies, respectively. The rabbit was anaesthetised using isoflurane and euthanised by intramuscular injection of pentobarbitone overdose. Blood was collected by cardiac puncture and allowed to rest at room temperature for two hours then 4°C overnight. Serum was extracted, added to an equal volume of 100% sterile glycerol (by autoclaving, 2.1.2) and stored in aliquots at -20°C until use.

3.3 RESULTS

3.3.1 Cloning of CDS5 (PGP3) in E. coli expression vectors

CDS5 encoding PGP3 derived from *C. trachomatis* pSW2 (serovar E) and pLGV440 (serovar L1) was amplified by PCR and cloned into expression vectors pRSET-A, pGEX-4T-1 and pGEX-6P-1 (3.2.1). Ampicillin-resistant recombinant clones were then screened by analysing their size against empty/uncloned vectors using agarose gel electrophoresis (2.4.2). All recombinant clones were verified by sequencing (2.6.4) prior to transformation into BL21 (DE3) pLysS *E. coli* for subsequent protein expression.

3.3.2 Analysis of expression and purification of his-tagged proteins

We purified recombinant PGP3 (derived from *C. trachomatis* pLGV440 and pSW2) and 3C (nS6) mouse norovirus protease initially expressed as fusion proteins with an N-terminal polyhistidine (x6) tag (3.2.2.1). Unless otherwise stated, all samples analysed by SDS-PAGE were analysed on 12.5% SDS gels with 10 µl PageRuler Prestained Protein Ladder. Samples were prepared by boiling and denaturing 30 µl aliquots in 10 µl 4x SDS loading buffer (2.10). 10 µl of prepared samples were loaded into the gel wells and any excess samples were frozen at -20°C.

3.3.2.1 His-PGP3

BL21 (DE3) pLysS *E. coli* cells transformed with recombinant plasmids pRSETA-LGV440-CDS5 (lane 3, Figure 3.5A) and pRSETA-SW2-CDS5 (lane 3, Figure 3.5B) (3.3.1) were induced with IPTG (2.8.4) for recombinant protein expression. The cells were lysed using BugBuster Master Mix (2.8.5.2) and the lysed fractions were analysed for the presence of his-tagged PGP3 (Figure 3.5A and 3.5B). Recombinant his-tagged PGP3 analysed by SDS-PAGE was present as a 35 kDa band consisting of the PGP3 protein (28 kDa) and the bound his-tag (7 kDa). His-tagged PGP3 derived from *C. trachomatis* pLGV440 was observed mainly in the insoluble cell pellet debris fraction following centrifugation (lane 4, Figure 3.5A) in comparison to the supernatant (lane 5, Figure 3.5A). In contrast, his-tagged PGP3 derived from pSW2 was observed as a soluble protein in the supernatant (lane 5, Figure 3.5B). Very little His-PGP3 was lost during the wash steps after cell lysis (lanes 6-9, Figure 3.5A and 3.5B).

His-PGP3 derived from pLGV440 and pSW2 were then purified using Probond Nickel-Chelating Resin (3.2.2.1) and eluted using imidazole (lanes 6-10, Figure 3.6A and 3.6B) using the buffers in Table 3.4. Despite the optimisation of purification conditions such as the volume of the nickel resin, some his-PGP3 derived from pLGV440 and pSW2 did not bind to the nickel-chelating resin and was lost during the wash steps (lanes 3-5, Figure 3.6A and 3.6B). His-tagged PGP3 derived from pSW2 required further elution steps with imidazole to obtain purified protein without non-specific products that had co-purified in previous steps (lanes 11-14, Figure 3.6B). The eluted fractions shown in lanes 6-10 (His-PGP3 derived from pLGV440, Figure 3.6A) and lanes 11-14 (His-PGP3 derived from pSW2, Figure 3.6B) were pooled for dialysis against PBS (2.9.3) before quantification using a protein assay kit (2.10.6).

Figure 3.5. Polyacrylamide gel analysis of recombinant His-tagged PGP3 following induction in an *E. coli* expression system and subsequent cell lysis

Recombinant PGP3 derived from *C. trachomatis* pLGV440 **[A]** and pSW2 **[B]** both expressed as fusion proteins with N-terminal polyhistidine tags (His-PGP3) were expressed in an *E. coli* expression system for 4 hours after induction with 1 mM IPTG and lysed using BugBuster Master Mix (BBMM). All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: non-induced (0 hr) and induced (4 hours, **lane 3**) cells taken from 1 ml of cell culture resuspended in 200 µl PBS; cell pellet debris resuspended in 5 ml 10% BBMM (**lane 4**) and supernatant (10 ml, **lane 5**) after lysis and centrifugation; **Lanes 6-9**: cell debris pellet following resuspension in 10 ml PBS and centrifugation; **Lane 10**: cell debris pellet following resuspension in 10 ml PBS].

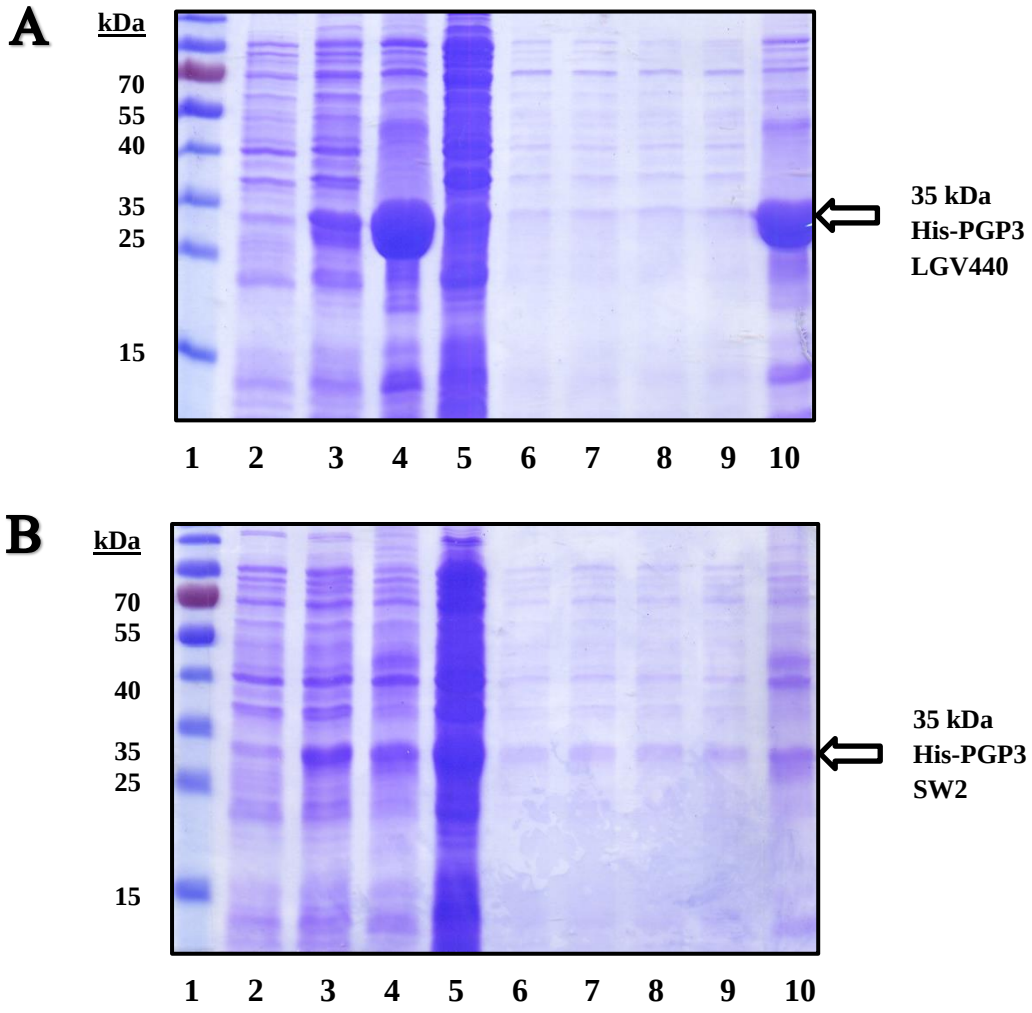
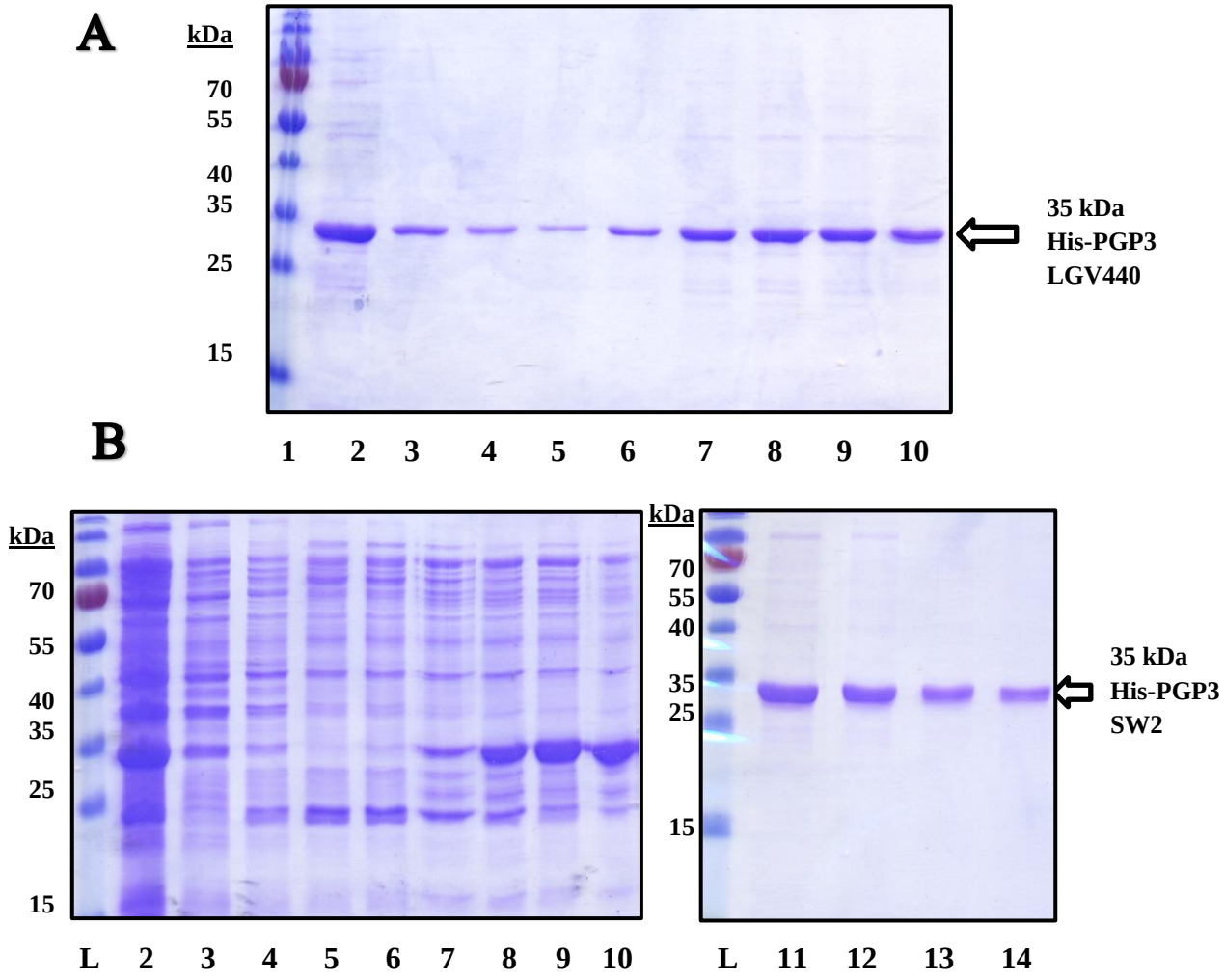


Figure 3.6. Polyacrylamide gel analysis of recombinant His-tagged PGP3 following purification using affinity chromatography

Extracts containing recombinant His-tagged PGP3 derived from *C. trachomatis* pLGV440 **[A]** and pSW2 **[B]** were bound to Probond Nickel-Chelating Resin for purification using affinity chromatography. All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**L**: molecular weight standards; **Lane 2**: protein-containing fractions following cell lysis resuspended in 10 ml binding buffer containing 10 mM imidazole; **Lane 3:** flow-through after binding to resin; **Lanes 4-5**; flow-through after washes in 4 ml wash buffers containing increasing concentrations of 25-50 mM imidazole; **Lanes 6-14**: purified His-PGP3 eluted in 1 ml fractions with elution buffer containing 250 mM imidazole].



3.3.2.2 His-3C (ns6) protease

The non-structural mouse norovirus protease 3C (nS6) was chosen as a negative control for the his-tagged PGP3 proteins to observe any potential reaction between the histidine tag and sera that may result in a false-positive signal in an ELISA or Western blot. DNA encoding the hydrophilic region of the 3C protease was previously cloned into the pRSET-A vector and transformed into competent BL21 (DE3) pLysS *E. coli* (by Dr Omar Salim). Cells were cultured, induced with IPTG (2.8.4) and lysed with BugBuster Master Mix (2.8.5.2) as described. Recombinant his-tagged 3C is observed as a 19 kDa protein consisting of his-tag (7 kDa) and the bound 3C protein (12 kDa). Following centrifugation, the recombinant his-tagged 3C protein was observed in the insoluble cell debris pellet (lane 4, Figure 3.7). Some expression was also observed before induction with IPTG (lane 2, Figure 3.7). A dimer of the 3C at approximately 38 kDa can also be observed in the pellet fraction (marked by the upper arrow).

His-tagged 3C protein was expressed and purified using the same method as his-tagged PGP3 derived from pLGV440, as described in 3.2.2.1. His-3C was purified using Probond Nickel-Chelating Resin (3.2.2.1) and eluted (lanes 6-10, Figure 3.8) using the buffers in Table 3.4. Some unbound his-tagged 3C protein was observed in the flow through and wash fractions (lanes 3-5, Figure 3.8) prior to elution with imidazole (lanes 6-10, Figure 3.8). The dimerization of the 3C protease was observed in these fractions. The eluted fractions shown in lanes 6 to 10 were pooled together for dialysis against PBS (2.9.3) before quantification (2.10.6).

Figure 3.7. Polyacrylamide gel analysis of recombinant His-tagged mouse norovirus 3C protease following induction in an *E. coli* expression system and subsequent cell lysis

Recombinant mouse norovirus 3C protease expressed as a fusion protein with an N-terminal polyhistidine tag (His-3C) was expressed in an *E. coli* expression system for 4 hours after induction with 1 mM IPTG and lysed using BugBuster Master Mix (BBMM). All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: non-induced (0 hr) and induced (4 hours, **lane 3**) cells taken from 1 ml of cell culture resuspended in 200 µl PBS; cell pellet debris resuspended in 5 ml 10% BBMM (**lane 4**) and supernatant (10 ml, **lane 5**) after lysis and centrifugation; **Lanes 6-9**: cell debris pellet following resuspension in 10 ml PBS and centrifugation; **Lane 10**: cell debris pellet following resuspension in 10 ml PBS].

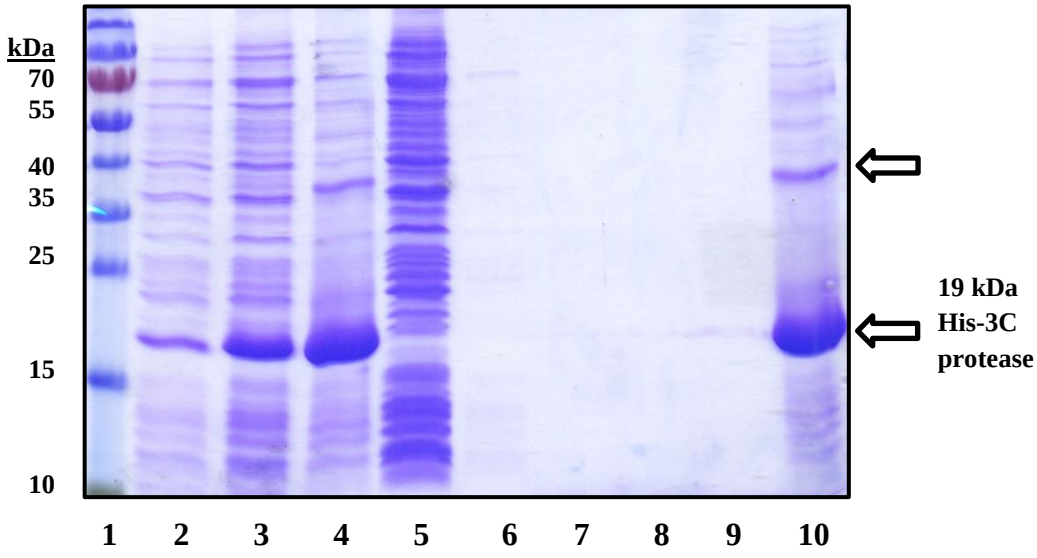
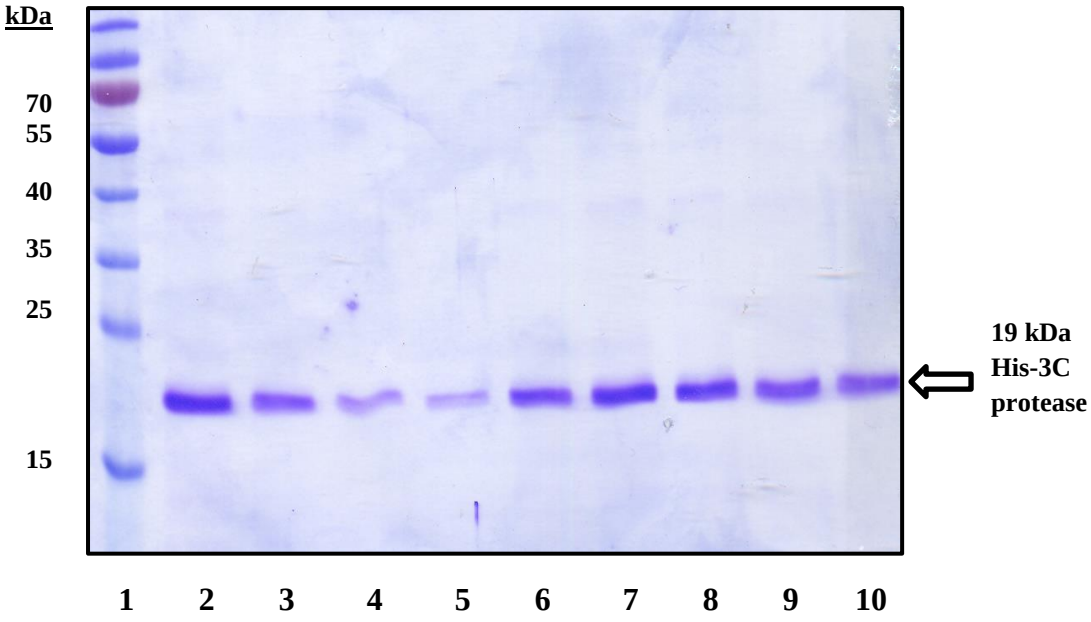


Figure 3.8. Polyacrylamide gel analysis of recombinant His-tagged 3C protease following purification using affinity chromatography

Extracts containing recombinant His-tagged mouse norovirus 3C protease was bound to Probond Nickel-Chelating Resin for purification using affinity chromatography. All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: protein-containing fractions resuspended in 10 ml binding buffer containing 10 mM imidazole; **Lane 3:** flow-through after binding to resin; **Lanes 4-5**; flow-through after washes in 4 ml wash buffers containing increasing concentrations of 25-50 mM imidazole; **Lanes 6-10**: purified His-3C mouse norovirus protease eluted in 1 ml fractions with elution buffer containing 250 mM imidazole].



3.3.3 Analysis of expression and purification of GST-tagged proteins

We also purified recombinant PGP3 expressed as a fusion protein with an N-terminal glutathione s-transferase (GST) tag. This 26 kDa tag is larger than the fusion tag expressed by the pRSET-A expression vector (7 kDa). However, GST is soluble and allows purification under native conditions using affinity chromatography. Unless otherwise stated, all samples analysed by SDS-PAGE were analysed on 12.5% SDS gels with 10 µl PageRuler Prestained Protein Ladder. Samples were prepared by boiling and denaturing 30 µl aliquots in 10 µl 4x SDS loading buffer (2.10). 10 µl of prepared samples were loaded into the gel wells and any excess samples were frozen at -20°C.

3.3.3.1 GST-PGP3

BL21 (DE3) pLysS *E. coli* transformed with recombinant plasmids pGEX4T1-SW2-CDS5 and pGEX4T1-LGV440-CDS5 (3.3.1) were induced with IPTG (2.8.4) for recombinant protein expression. The cells were lysed using BugBuster Master Mix (2.8.5.2) and the lysed fractions were analysed for the presence of GST-tagged PGP3 (Figure 3.9A and 3.8B). Recombinant GST-tagged PGP3 analysed by SDS-PAGE was present as a 54 kDa band consisting of the PGP3 protein (28 kDa) and the bound N-terminal GST-tag (26 kDa). GST-tagged PGP3 derived from *C. trachomatis* pLGV440 and pSW2 were both observed in the cell pellet and supernatant following centrifugation of the lysed cells (lane 5, Figure 3.9A and 3.9B).

GST-tagged PGP3 derived from pLGV440 and pSW2 were purified using glutathione sepharose 4B beads (3.2.2.2) and eluted using reduced glutathione (lanes 6-10, Figure 3.10A and 3.10B) and the buffers in Table 3.5. GST-tagged PGP3 that had not bound to glutathione sepharose beads was observed in the flow through (lane 3, Figure 3.10A and 3.10B). The GST-tagged PGP3 protein can be seen as a 54 kDa protein consisting of the PGP3 protein (28 kDa) and the glutathione s-transferase tag (26 kDa). During the elution steps, two unspecific and unidentified protein bands at around 26 and 40 kDa were observed during the purification of GST-PGP3 from pLGV440 and pSW2 (lanes 5-6, Figure 3.10A and 3.10B). The eluted fractions shown in lanes 8-10 (LGV440) and 7 to 10 (SW2) were pooled together for dialysis against PBS (2.9.3) before quantification using a protein assay kit (2.10.6).

Figure 3.9. Polyacrylamide gel analysis of recombinant GST-PGP3 following induction in an *E. coli* expression system and subsequent cell lysis

Recombinant PGP3 derived from *C. trachomatis* pLGV440 and pSW2 expressed as fusion proteins with N-terminal glutathione s-transferase (GST) tags (GST-PGP3) were expressed in an *E. coli* expression system for 4 hours after induction with 1 mM IPTG and lysed using BugBuster Master Mix (BBMM). All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: non-induced (0 hr) and induced (4 hours, **lane 3**) cells taken from 1 ml of cell culture resuspended in 200 µl PBS; cell pellet debris (**lane 4**) resuspended in 5 ml 10% BBMM and supernatant (10 ml, **lane 5**) after lysis and centrifugation].

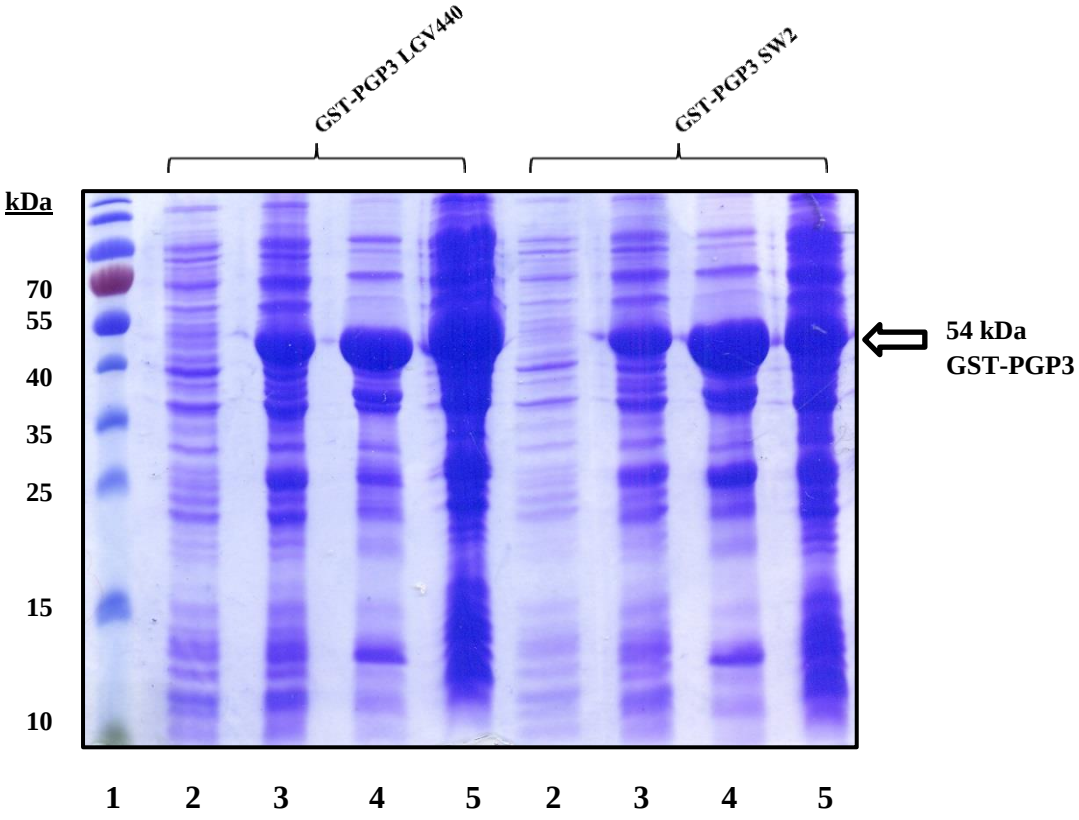
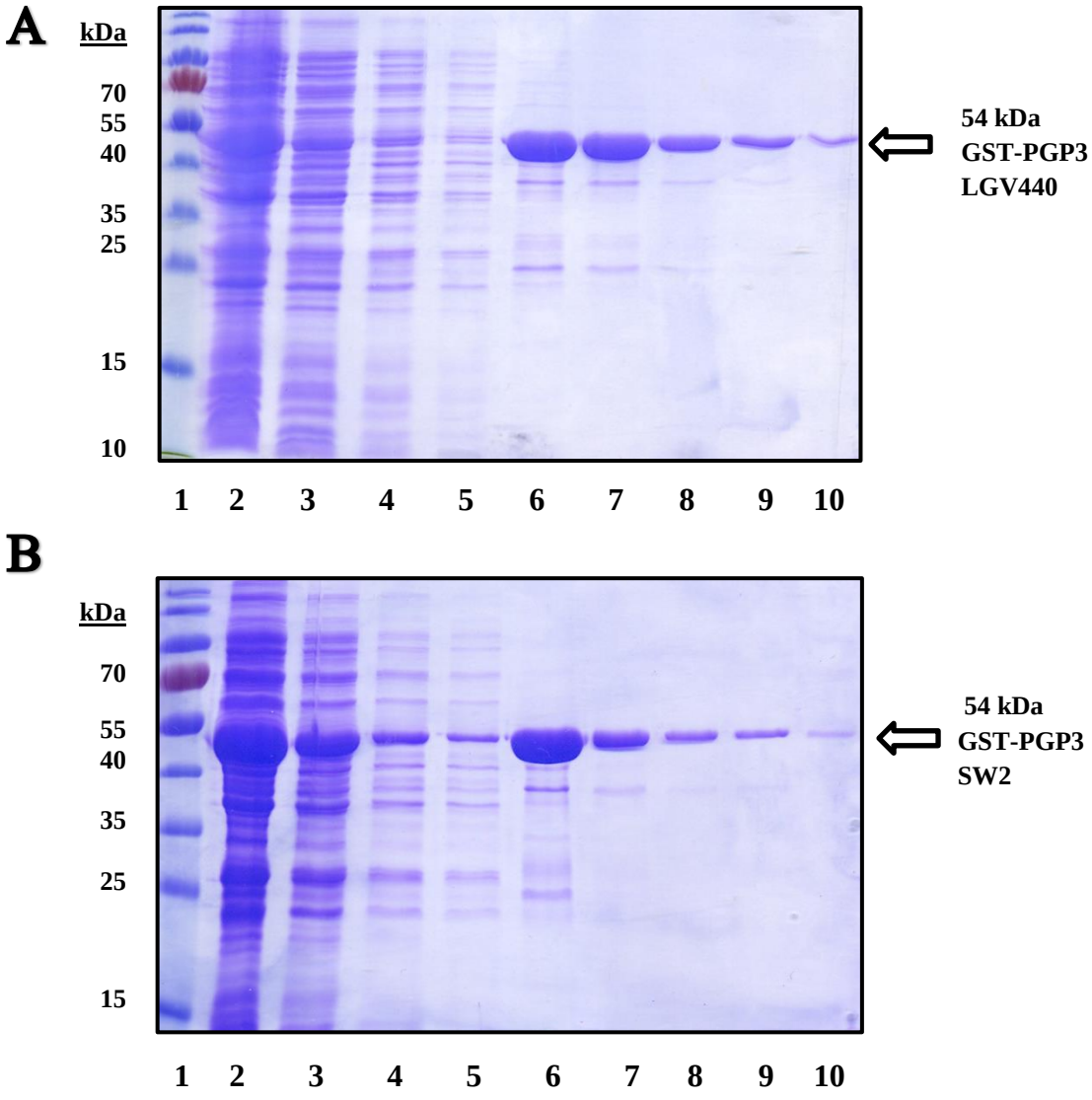


Figure 3.10. Polyacrylamide gel analysis of recombinant GST-PGP3 following purification using affinity chromatography

Extracts containing recombinant GST-PGP3 derived from *C. trachomatis* pLGV440 **[A]** and pSW2 **[B]** were bound to glutathione sepharose 4B resin for purification using affinity chromatography. All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: flow-through after binding to glutathione sepharose (10 ml); **Lanes 3-5**: flow-through after washes in 2 ml PBS; **Lanes 6-10**: purified GST-PGP3 eluted in 1 ml fractions with elution buffer containing 50 mM reduced glutathione].



3.3.3.2 GST

Glutathione s-transferase (GST) was used as a negative control to observe any potential reaction between the sera and the GST tag on the GST-tagged PGP3 fusion proteins that may result in a false-positive signal in an ELISA or Western blot. Competent BL21 (DE3) pLysS *E. coli* were transformed with empty/uncloned pGEX-4T-1 and pGEX-6P-1 expression vectors (2.8.2) for protein expression. GST expressed using the pGEX-4T-1 expression vector was used as a negative control for proteins expressed and purified as GST-tagged fusion proteins using the pGEX-4T-1 vector (3.2.2.2). GST expressed using the pGEX-6P-1 expression vector was used as a negative control for proteins initially expressed as a GST-tagged fusion protein from the pGEX-6P-1 that were subsequently cleaved using Prescission Protease (3.2.2.3). Both GST proteins differ only by eight amino acids due to the cleavage sites of thrombin and Prescission Protease. Transformed cells were cultured and induced with IPTG (2.8.4) and lysed with BugBuster Master Mix (2.8.5.2) as described. GST protein was observed in the supernatant following cell lysis and centrifugation (lane 5, Figure 3.11A and 3.11B).

The supernatant was added to glutathione sepharose 4B beads and purified using the same protocol as the GST-tagged PGP3 proteins in 3.2.2.2. Some unbound GST protein was observed in the flow through and wash preparations (lanes 2-5, Figure 3.12A and 3.12B). Bound GST was eluted with reduced glutathione buffer (lanes 6-10, Figure 3.12A and 3.12B). Some non-specific and unidentified protein bands were eluted with the GST proteins at approximately 15 and 20 kDa (lanes 6 and 7, Figure 3.12A and 3.12B). GST in lanes 7-10 (GST-4T-1) 8-10 (GST-6P-1) (Figure 3.12A and 3.12B) were pooled together for dialysis against PBS (2.9.3) before quantification (2.10.6).

Figure 3.11. Polyacrylamide gel analysis of recombinant glutathione s-transferase (GST) following induction in an *E. coli* expression system and subsequent cell lysis

Recombinant GST expressed from pGEX-4T-1 **[A]** and pGEX-6P-1 **[B]** expression vectors were expressed in an *E. coli* expression system for 4 hours after induction with 1 mM IPTG and lysed using BugBuster Master Mix (BBMM). All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: non-induced (0 hr) and induced (4 hours, **lane 3**) cells taken from 1 ml of cell culture resuspended in 200 µl PBS; cell pellet debris (**lane 4**) resuspended in 5 ml 10% BBMM and supernatant (10 ml, **lane 5**) after lysis and centrifugation].

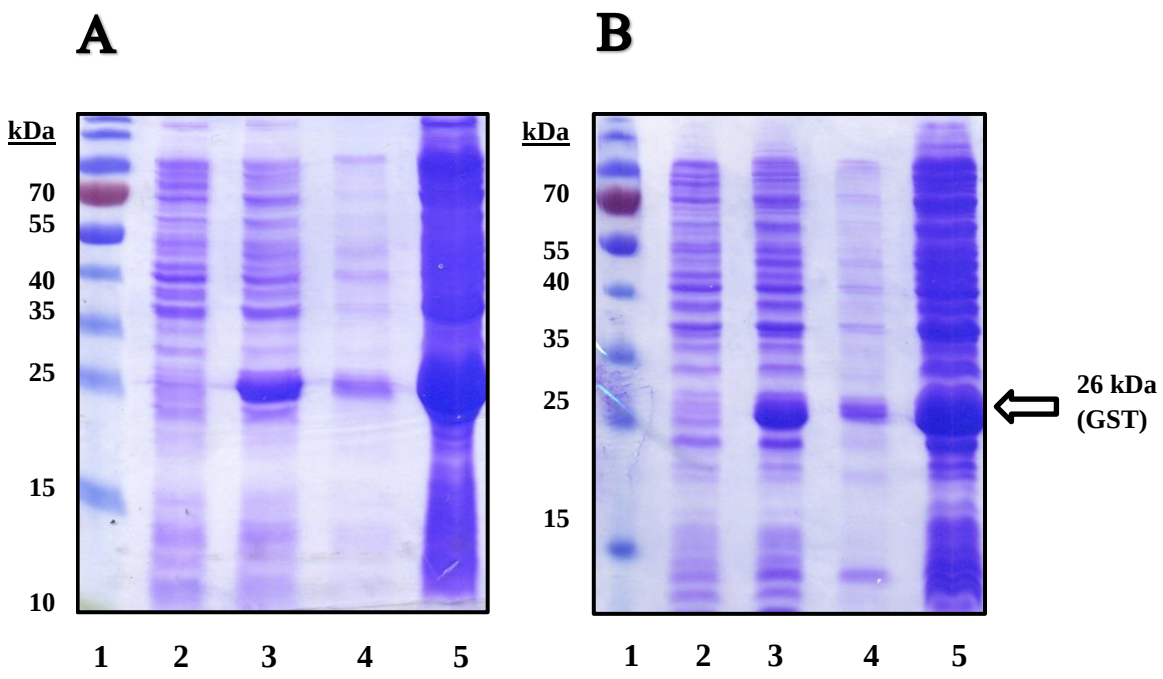
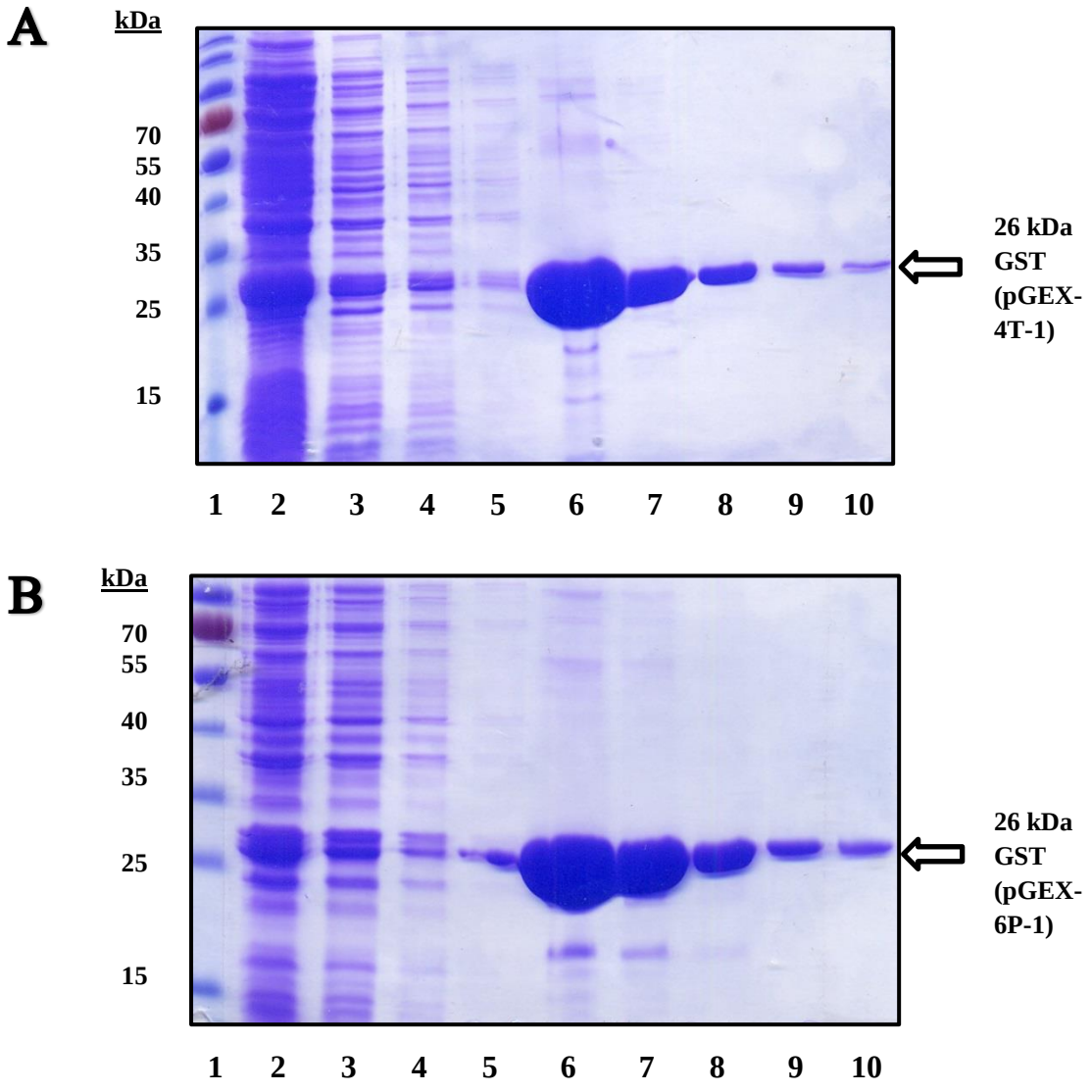


Figure 3.12. Polyacrylamide gel analysis of recombinant glutathione s-transferase (GST) following purification using affinity chromatography

Extracts containing recombinant GST derived from pGEX-4T-1 **[A]** and pGEX-6P-1 **[B]** expression vectors were bound to glutathione sepharose 4B resin for purification using affinity chromatography. All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: flow-through after binding to glutathione sepharose (10 ml); **Lanes 3-5**: flow-through after washes in 2 ml PBS; **Lanes 6-10**: purified GST-PGP3 eluted in 1 ml fractions with elution buffer containing 50 mM reduced glutathione].



3.3.3.3 Expression and purification of cleaved (untagged) PGP3

To investigate the possibility that the N-terminal GST fusion tag was affecting the sensitivity of our ELISAs (see chapter 5) or was affecting antibody recognition of PGP3 in Western blots, we purified untagged PGP3 purified initially as a GST-tagged fusion protein to be subsequently cleaved from the GST tag (3.2.2.3).

3.3.3.3-1 pGEX-4T-1 (Thrombin)

BL21 (DE3) pLysS *E. coli* cells transformed with the recombinant plasmids pGEX4T1-SW2-CDS5 and pGEX4T1-LGV440-CDS5 (3.3.1) were induced with IPTG (2.8.4) and lysed with BugBuster Master Mix (2.8.5.2) as described. GST-PGP3 was observed in the supernatant following cell lysis and centrifugation (lane 5, Figure 3.13A and 3.13B). The supernatant containing recombinant GST-PGP3 was added to glutathione sepharose 4B beads and unbound GST-PGP3 and washes were collected as described in 3.2.2.2.

We initially attempted to purify cleaved PGP3 derived from *C. trachomatis* pSW2 and pLGV440 produced initially as a fusion protein using the pGEX-4T-1 expression vector (2.8.2). Purifying PGP3 cleaved directly from GST-PGP3 bound to the GST column using a Thrombin CleanCleave Kit (Sigma-Aldrich) would have allowed GST to remain bound to the beads and not co-purify with the cleaved PGP3. As thrombin from this kit is bound to agarose beads, thrombin can be removed from solution by centrifugation. This method would have allowed the purification of cleaved PGP3 without GST or thrombin. For the enzymatic cleavage, the manufacturer's instructions for cleavage were followed but this was unsuccessful as no cleaved PGP3 was observed (data not shown). As the manufacturer recommended initially purifying the fusion protein first using reduced glutathione and then attempt to cleave the protein from GST using the kit, we made another attempt to obtain cleaved PGP3 from purified fusion protein. As the GST-PGP3 fusion protein was no longer bound to the column, this method involved GST co-purifying with the cleaved PGP3 protein. However, this method had also been unsuccessful for both GST-PGP3 proteins derived from pLGV440 and pSW2 proteins (data not shown).

3.3.3.3-2 pGEX-6P-1 (Prescission Protease)

As purification cleaved PGP3 using the Thrombin CleanCleave Kit was unsuccessful (3.3.2.3-1), we attempted to cleave PGP3 from the GST tag using Prescission Protease (3.2.2.3). BL21 (DE3) pLysS *E. coli* cells transformed with the recombinant plasmids pGEX6P1-SW2-CDS5 and pGEX6P1-LGV440-CDS5 (3.3.1) were induced with IPTG (2.8.4) and lysed with BugBuster Master Mix (2.8.5.2) as described. GST-PGP3 was observed in the supernatant following cell lysis and centrifugation (lane 5, Figure 3.13).

The supernatants were added to glutathione sepharose 4B beads and unbound GST-PGP3 and washes were collected (lanes 3-6, Figure 3.14A and 3.14B). Cleaved PGP3 was collected using Prescission Protease as described (3.2.2.3) and analysed (Figure 3.14A and 3.14B). Some unbound GST-PGP3 derived from *C. trachomatis* pLGV440 and pSW2 was observed in the flow through and wash steps (lanes 3-7, Figure 3.14A and 3.14B). Cleavage of PGP3 from bound GST was successful using Prescission Protease (lane 8, Figure 3.14A and lanes 8-9, 3.14B) and no GST was observed. A weak non-specific band at 54 kDa was observed which is likely to be residual uncleaved GST-PGP3. Another non-specific band at around 40 kDa was also observed. However, this band is also present in the elution steps of GST-PGP3 derived from both pLGV440 and pSW2 even after multiple purification optimisation methods, as seen in lane 6 in Figure 3.10A and 3.10B. Some cleaved PGP3 was observed in the sepharose 4B bead mixture even after purification and cleavage (lane 10, Figure 3.14A and 3.14B). Attempts to obtain this protein using multiple washes in cleavage buffer (Table 3.5) were not successful (data not shown). Purified cleaved 28 kDa PGP3 protein was dialysed overnight against PBS (2.9.3) before quantification (2.10.6). PGP3 from *C. trachomatis* pSW2 was incubated with Prescission Protease for 16 hours as this was an optimised protocol to achieve a higher yield of protein that would be later used for rabbit immunisation (3.2.3). Cleaved PGP3 derived from both pSW2 and pLGV440 was achieved after 4 hours but PGP3 from pLGV440 was not used in any ELISA or immunisation experiments and so the protocol was not optimised.

Figure 3.13. Polyacrylamide gel analysis of recombinant GST-PGP3 following induction in an *E. coli* expression system and subsequent cell lysis

Recombinant PGP3 derived from *C. trachomatis* pLGV440 and pSW2 expressed with N-terminal GST tags (GST-PGP3) using the pGEX-6P-1 expression vector were expressed in an *E. coli* expression system for 4 hours after induction with 1 mM IPTG and lysed using BugBuster Master Mix (BBMM). All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. [**Lane 1**: molecular weight standards; **Lane 2**: non-induced (0 hr) and induced (4 hours, **lane 3**) cells taken from 1 ml of cell culture resuspended in 200 µl PBS; cell pellet debris (**lane 4**) resuspended in 5 ml 10% BBMM and supernatant (10 ml, **lane 5**) after lysis and centrifugation].

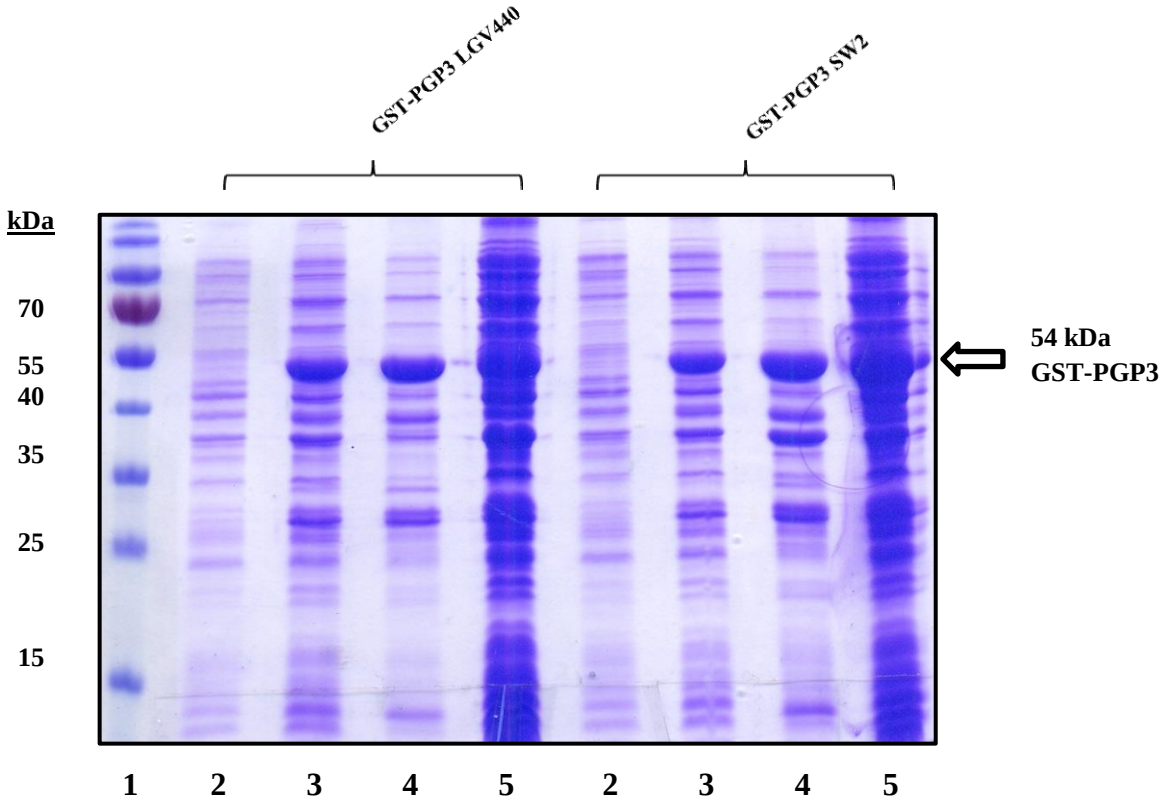
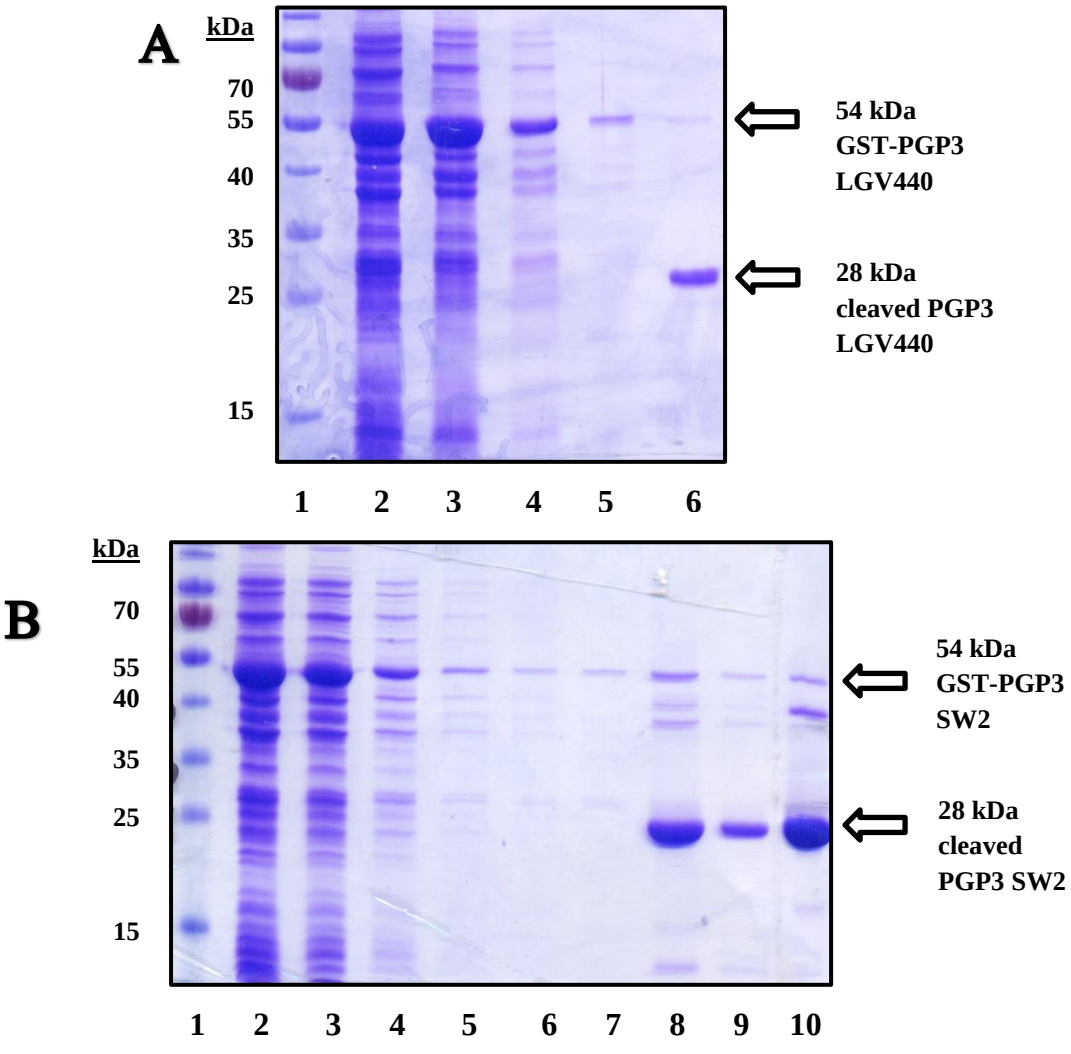


Figure 3.14. Polyacrylamide gel analysis of recombinant PGP3 enzymatically cleaved from the GST tag following purification using affinity chromatography

Extracts containing recombinant GST-PGP3 derived from *C. trachomatis* pLGV440 **[A]** and pSW2 **[B]** were bound to glutathione sepharose 4B resin for subsequent cleavage from the GST tag using Prescission Protease. All samples were denatured in SDS buffer (2% SDS, 1.25% BME and heat boiled for 5 minutes). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels and visualised using Coomassie brilliant blue staining. **[A]** and **[B]** [**Lane 1**: molecular weight standards; **Lane 2**: flow-through after binding to glutathione sepharose (10 ml); **Lanes 3-4:** flow-through after washes in 2 ml PBS; **Lane 5:** flow through after washes in 2 ml cleavage buffer; **Lane 6 [A only]:** cleaved PGP3 (5 ml) after 4 hours incubation with Prescission Protease]. **[B only] Lanes 5-7:** flow through after washes in 2 ml cleavage buffer; cleaved PGP3 after 4 hours (1 ml, **lane 8**) and 16 hours (5 ml, **lane 9**) incubation with Prescission Protease; **Lane 10:** glutathione sepharose resin resuspended in 1 ml cleavage buffer].

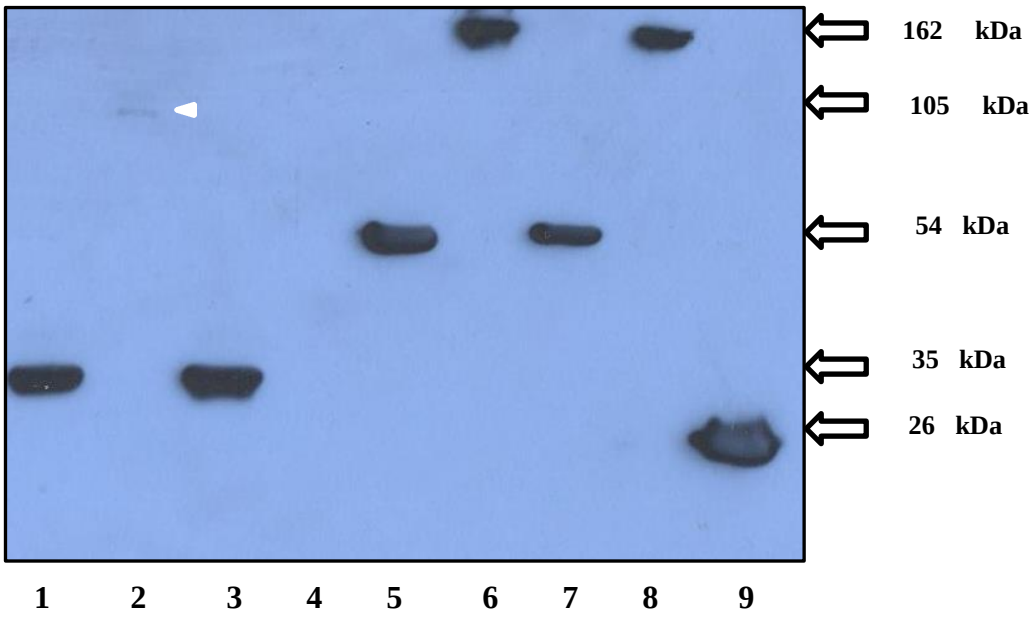


3.3.4 Detection of recombinant his- and GST-tagged PGP3 by Western blot

The potential trimerisation of our recombinant PGP3 and his/GST-PGP3 proteins were investigated using positive control antibodies in a Western blot. All purified recombinant proteins (GST- and his- tagged PGP3 and cleaved PGP3 derived from *C. trachomatis* pSW2 and pLGV440, his-3C protease and GST) were analysed by SDS-PAGE on 12.5% gels (2.10), transferred to a PVDF membrane (2.10.7), incubated with mouse monoclonal anti-GST/his IgG at 1:500 and detected using anti-mouse IgG-HRP at 1:2000 (2.10.8.1). Membranes were then visualised using an ECL kit (2.10.8.3). Native and denatured structures of proteins were observed as described (2.10.2). It was discovered that the addition of SDS to the running buffers and polyacrylamide gels aided the migration of PGP3 through the gel but did not affect its native trimeric structure or antibody recognition. All monomeric structures of recombinant his-tagged PGP3 and GST-tagged PGP3 proteins were detected (lanes 1 and 3, and 5 and 7, respectively, Figure 3.15). GST was only treated with denaturing SDS loading buffer in this experiment (lane 9, Figure 3.15) as only monomeric GST was observed when treated with native loading buffer in SDS-PAGE (2.10.2) in a previous Western blot experiment (data not shown). Trimers of GST-PGP3 derived from both *C. trachomatis* pSW2 and pLGV440 were observed at approximately 162 kDa (lanes 6 and 8, Figure 3.15). In contrast, a weak band at approximately 105 kDa was observed in lane 2, suggesting partial trimerisation of his-PGP3 derived from pSW2 (white arrow). However, no monomeric his-PGP3 was detected in the same lane. No trimerisation of his-PGP3 derived from pLGV440 was observed (lane 4, Figure 3.15). However, similar to his-PGP3 derived from pSW2, no monomeric his-PGP3 from pLGV440 was detected in the same lane.

Figure 3.15. Analysis of trimerisation of recombinant N-terminal GST- and polyhistidine tagged- PGP3 by western blot.

1 µg aliquots of his- and GST- tagged recombinant PGP3 derived from *C. trachomatis* pSW2 and pLGV440 and GST were analysed by SDS-PAGE on 12.5% gels, transferred to a PVDF membrane, blocked with 10% skimmed milk, incubated with mouse monoclonal anti-GST/his IgG at 1:500 and anti-mouse IgG-HRP at 1:2000 and then visualised using an ECL kit. Samples of recombinant protein were denatured by treating in SDS buffer (2% SDS, 1.25% BME) and heating to 100°C for 5 minutes, or left untreated to observe native structures. [**Lane 1**: His-PGP3 SW2 (treated); **Lane 2**: His-PGP3 SW2 (untreated); **Lane 3**: His-PGP3 LGV440 (treated); **Lane 4**: His-PGP3 LGV440 (untreated); **Lane 5**: GST-PGP3 SW2 (treated); **Lane 6**: GST-PGP3 SW2 (untreated); **Lane 7**: GST-PGP3 LGV440 (treated); **Lane 8**: GST-PGP3 LGV440 (untreated); **Lane 9**: GST (treated)].



3.3.5 Detection of recombinant PGP3 using rabbit polyclonal anti-PGP3 antisera

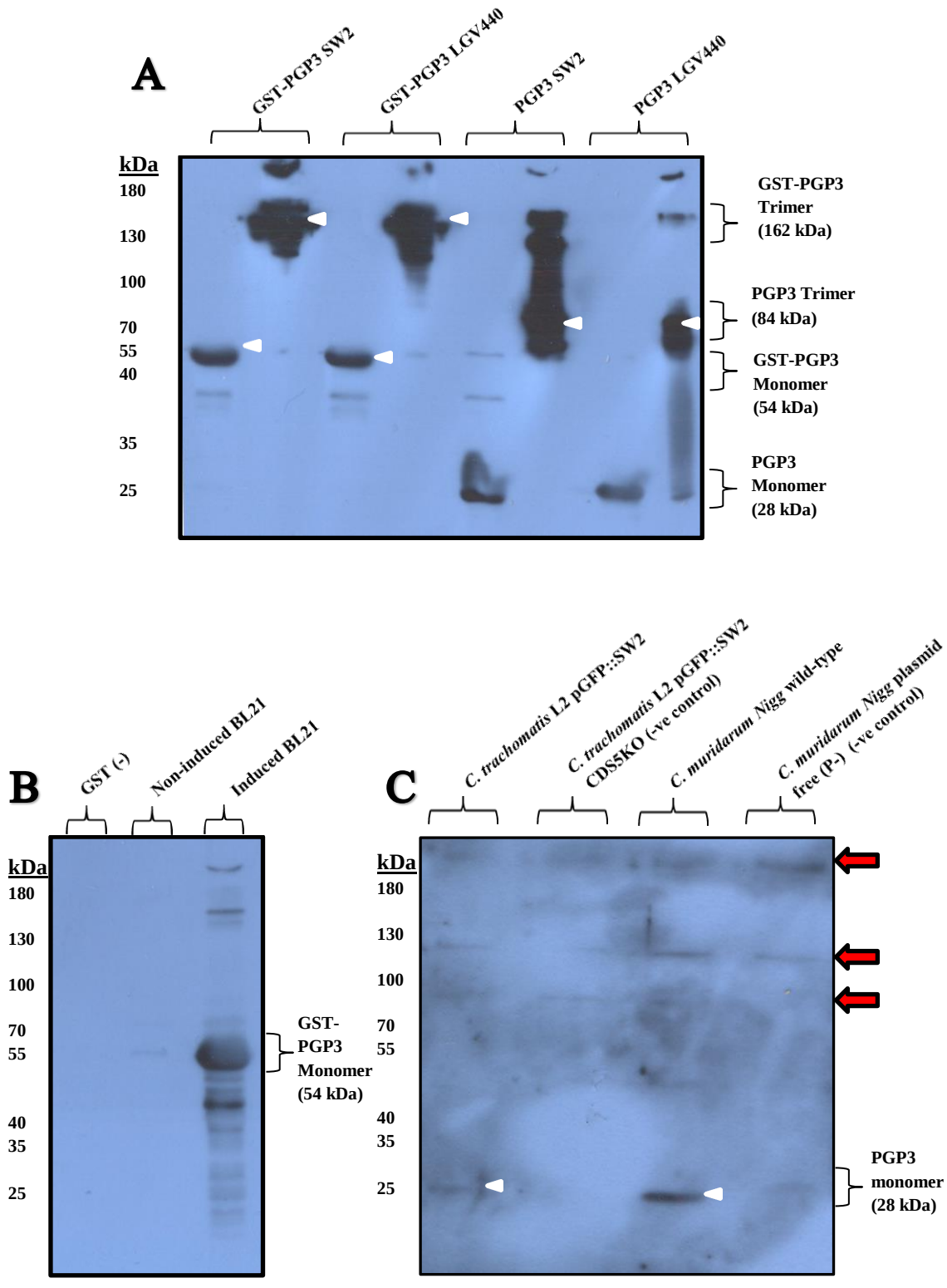
Purified cleaved PGP3 from *C. trachomatis* pSW2 was used to immunise one female New Zealand white rabbit for the production of polyclonal anti-PGP3 SW2 antisera, as described (3.2.3), for use as a positive control antibody in ELISAs and Western blots. The rabbit was given four fortnightly 50 µg intramuscular injections using Freund's Complete (x1)/Incomplete (x3) adjuvant. Samples of sera were collected before the first and before the forth immunisation to confirm the absence and presence of anti-PGP3 antibodies by Western blot, respectively (data not shown).

Using Western blot, we tested the reactivity of our anti-PGP3 antisera with recombinant GST-tagged PGP3, recombinant PGP3 that had been cleaved from the GST tag, and PGP3 in chlamydial elementary bodies. 30 µl aliquots of recombinant GST-PGP3 derived from pSW2 and pLGV440, cleaved PGP3 derived from pSW2 and pLGV440, GST, purified elementary bodies of *C. trachomatis* L2 transformed with pGFP::SW2 and purified *C. muridarum* Nigg WT were denatured in 10 µl 4x SDS loading buffer (2.10.2), analysed by SDS-PAGE in 10 µl aliquots (2.10) and transferred to a PVDF membrane (2.10.7). We also included *C. trachomatis* L2 transformed with pGFP::SW2 containing a CDS5 deletion (CDS5KO) and *C. muridarum* Nigg P- as negative controls as these strains do not express PGP3. We also analysed 10 µl aliquots of BL21 (DE3) pLysS *E. coli* transformed with the recombinant pGEX4T1-CDS5-SW2 (3.3.1) expression plasmid (non-induced and induced for 4 hours with 1mM IPTG, 2.8.4) to observe any non-specific reaction to non-specific products that had co-purified during purification steps (3.2.2). For recombinant proteins and BL21 (DE3) pLysS *E. coli*, anti-PGP3 antiserum was used at a 1:2000 dilution. For chlamydial cultures, anti-PGP3 antiserum was used at a 1:100 dilution. Membranes were then incubated with anti-rabbit IgG-HRP IgG and visualised using an ECL kit (2.10.8.3). Anti-PGP3 antisera reacted with both monomeric and trimeric GST-PGP3 and PGP3 derived from pSW2 and pLGV440 (all lanes, Figure 3.16A). A reaction was also observed to the induced culture of BL21 (DE3) pLysS *E. coli* transformed with the pGEX4T1-CDS5-SW2 expression plasmid (lane 3, Figure 3.16B). No reaction was observed to the non-induced culture of transformed BL21 *E.coli* (lane 2, Figure 3.16B). No reaction was observed to the monomeric GST negative control (lane 1, Figure 3.16B). A weak band at approximately 28 kDa was observed in the *C. trachomatis* L2 pGFP::SW2 and *C. muridarum* WT strains (lanes 1 and 3, Figure 3.16C, respectively, marked by white arrows),

corresponding with the expected size of the monomeric PGP3 protein (28 kDa). No 28 kDa band was observed in the *C. trachomatis* L2 pSW2-CDS5KO or *C. muridarum* P- strains (lanes 2 and 4, Figure 3.16C, respectively). However, some mild reactions to non-specific bands were observed in all lanes at approximately 180, 70 and 50 kDa (marked by red arrows, Figure 3.16C).

Figure 3.16. Detection of recombinant PGP3 trimerisation and by polyacrylamide gel electrophoresis using rabbit anti-PGP3 antisera.

Recombinant PGP3 derived from *C. trachomatis* pSW2 and pLGV440 both expressed as fusions with an N-terminal GST tag were purified from an *E. coli* expression system in which the tag was retained (GST-PGP3) or subsequently cleaved (PGP3). 1 µg samples of purified recombinant protein were denatured by treating in SDS buffer (2% SDS, 1.25% BME) and heating to 100°C for 5 minutes, or left untreated to observe native structures. **[A]** PGP3 and GST-PGP3 trimerisation were visualised using a rabbit polyclonal antibody to PGP3 (pAb anti-PGP3; 1:2000) and goat anti-rabbit IgG-HRP (1:2000). pAb rabbit anti-PGP3 was produced by immunisation using cleaved PGP3 derived from *C. trachomatis* pSW2. Trimers of GST-PGP3 and PGP3 are marked by white arrows. **[B]** Rabbit pAb anti-PGP3 was blotted at 1:2000 against GST-PGP3 expressed in whole cells of BL21 (DE3) pLysS *E. coli* induced for 4 hours. **[C]** Rabbit pAb anti-PGP3 was blotted at 1:2000 against purified chlamydial elementary bodies (*C. trachomatis* L1 pGFP::SW2, *C. trachomatis* L2 pGFP::SW2 (CDS5 knock-out), *C. muridarum* Nigg wild-type, *C. muridarum* Nigg plasmid-free). Positive reactions to PGP3 are marked by the white arrows.


A
GST-PGP3 SW2
GST-PGP3 LGV440
PGP3 SW2
PGP3 LGV440
kDa
180
130
100
70
55
40
35
25
GST-PGP3 Trimer (162 kDa)
PGP3 Trimer (84 kDa)
GST-PGP3 Monomer (54 kDa)
PGP3 Monomer (28 kDa)
B
GST (-)
Non-induced BL21
Induced BL21
kDa
180
130
100
70
55
40
35
25
GST-PGP3 Monomer (54 kDa)
C
C. trachomatis L2 pGFP::SW2
C. trachomatis L2 pGFP::SW2 CDS5KO (-ve control)
C. muridarum Nigg wild-type
C. muridarum Nigg plasmid free (P-) (-ve control)
kDa
180
130
100
70
55
40
35
25
PGP3 monomer (28 kDa)

3.4 DISCUSSION

In this chapter, the purification of recombinant PGP3 derived from *C. trachomatis* plasmids pSW2 (serovar E) and pLGV440 (serovar L1) as fusion proteins with N-terminal polyhistidine or glutathione s-transferase (GST) tags was described. Recombinant PGP3 derived from *C. trachomatis* pSW2 was later enzymatically cleaved from the GST tag and purified for the production of rabbit polyclonal anti-PGP3 antisera by immunisation.

We initially purified recombinant PGP3 using an N-terminal polyhistidine tag produced using the expression vector pRSET-A and this tag allowed native purification using affinity chromatography. Furthermore, the expressed N-terminal polyhistidine tag is only 7 kDa in size and it was anticipated that the small tag would have minimal effect on the native structure of the 28 kDa recombinant PGP3 protein. However, some differences were observed during the purification of recombinant PGP3 from pSW2 (his-PGP3 SW2) and pLGV440 (his-PGP3 LGV440). After expression, lysis and centrifugation of lysed BL21 (DE3) pLysS *E. coli*, recombinant PGP3 derived from pSW2 was observed in the supernatant and PGP3 derived from pLGV440 was observed in the cell debris pellet. According to the manufacturer's protocol, cell lysis using BugBuster Master Mix releases soluble proteins into the supernatant after centrifugation. As the tag contains six histidine residues which possess an overall positive charge, and recombinant PGP3 has been reported to be in the soluble factions after protein induction and cell lysis (74, 75, 167), it was expected that both his-PGP3 SW2 and his-PGP3 LGV440 would be observed in the supernatant after cell lysis. Furthermore, using Western blot assay it was revealed that his-PGP3 SW2 was able to trimerise whereas his-PGP3 LGV440 was not. The polypeptide of PGP3 derived from *C. trachomatis* pSW2 and pLGV440 differ by nine amino acids (126) and these variations are reflected in the different isoelectric points (pI) of PGP3 between serovars E and L1 (4.34 and 4.57, respectively). This may account for the differences observed after cell lysis and of his-PGP3 SW2 and his-PGP3 LGV440. However, the trimerisation of recombinant PGP3 derived from *C. trachomatis* pLGV440 that had been cleaved from the N-terminal fusion tag was later observed by Western blot assay. Therefore, it was concluded that the differences in native structure observed between his-PGP3 SW2 and his-PGP3 LGV440 were either due to the polyhistidine tag or due to suboptimal protein purification conditions (e.g. over-expression of protein leading to

inclusion body formation). These Western blotting experiments were conducted after the his-PGP3 proteins were assessed for their potential to detect anti-PGP3 antibodies in patient sera by ELISA as it was not yet known that the recombinant PGP3 proteins were able to trimerise, as described further in chapter four.

Recombinant PGP3 derived from *C. trachomatis* pSW2 and pLGV440 was then expressed and purified using N-terminal GST tags. GST carries an overall positive charge and is 26 kDa is size. Although the GST tag is larger than the his-tag expressed by the pRSET-A vector (7 kDa), the native purification of recombinant PGP3 expressed and purified with an N-terminal GST tag has been described (74, 118, 167) and all proteins were observed in the supernatant after expression and cell lysis. These studies include the expression and purification of GST-PGP3 derived from *C. trachomatis* serovars L1 and D. Despite this, although some protein was observed in the cell debris pellet following cell lysis, GST-PGP3 derived from *C. trachomatis* pSW2 (GST-PGP3 SW2) and pLGV440 (GST-PGP3 LGV440) were mostly observed as soluble proteins in the supernatant after cell lysis. Furthermore, in contrast to his-PGP3 SW2 and his-PGP3 LGV440, trimerisation of both GST-PGP3 SW2 and GST-PGP3 LGV440 proteins was observed using Western blot assay. Trimerisation of recombinant GST-PGP3 and PGP3 has been described (74, 124, 125). In contrast, all recombinant GST was observed in the supernatant after cell lysis and no trimerisation or dimerisation was observed by Western blot assay. An explanation for the observation of some GST-PGP3 in the cell debris pellet was that the protein was over-expressed and inclusion bodies had formed. However, the recombinant GST protein was expressed and purified using the same protocol. Interestingly, the yield of GST obtained through purification was approximately 10-fold higher than that obtained for the GST-PGP3 proteins. This may be due to the loss of some GST-PGP3 as flow-through during the binding of GST-PGP3 to the glutathione sepharose 4B beads during purification. An explanation for this is that binding of the GST-PGP3 to the beads may have been blocked by PGP3 trimer formation. However, no trimerisation or dimerisation of GST was observed by Western blot, suggesting that trimerisation of the GST-PGP3 was due to the trimerisation of PGP3. This may also explain why cleavage of PGP3 from the GST tag using thrombin agarose beads had failed. Despite this, a high yield of GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 was achieved and PGP3 SW2 and PGP3 LGV440 were able to be cleaved from the GST tag using Prescission Protease. The

reproducibility of the protocol used for the expression and purification of GST-PGP3 and PGP3 cleaved from the GST tag has also been demonstrated as at least five batches of each recombinant protein have been expressed and purified.

Trimerisation of PGP3 SW2 and PGP3 LGV440 was also observed by Western blot assay. This was observed using a rabbit polyclonal anti-PGP3 positive control antibody was that produced using purified recombinant PGP3 SW2 as the antigen. PGP3 derived from *C. trachomatis* pSW2 was chosen for immunisation as this is from the common urogenital serovar, E. As a negative control, this antibody was also assayed against GST and whole cells of non-induced BL21 (DE3) pLysS *E. coli* transformed with the recombinant pGEX6P1-CDS5-SW2 expression vector. This was to observe any potential reactivity to non-specific products that may have co-purified with recombinant PGP3. These non-specific products would have then been prepared for immunisation to produce anti-PGP3 antisera. However, as no reaction was observed to GST and only a weak reaction was observed to GST-PGP3 in the non-induced cell culture, this suggests that the recombinant PGP3 SW2 used for immunisation was pure and contained no or very little non-specific impurities. The rabbit polyclonal anti-PGP3 antisera was also able to detect monomeric and trimeric cleaved PGP3 and GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440. Furthermore, using Western blot, the rabbit anti-PGP3 antisera reacted to PGP3 expressed in *C. trachomatis* L1 transformed with pGFP::SW2 and wild-type *C. muridarum* Nigg. This suggests that antibodies produced to PGP3 in humans and mice infected with *C. trachomatis* and *C. muridarum*, respectively, may react to the recombinant PGP3 in ELISAs and Western blots.

3.4.1 Conclusions

- Recombinant His-tagged PGP3 derived from *C. trachomatis* pLGV440 (serovar L1) is insoluble during purification using affinity chromatography and no trimerisation is observed in a Western blot.
- Recombinant His-PGP3 derived from *C. trachomatis* pSW2 (serovar E) is found in the soluble fraction and trimerisation is observed in a Western blot.
- Recombinant GST-tagged PGP3 derived from *C. trachomatis* pLGV440 and pSW2 are able to trimerise as observed by Western blot.
- Recombinant GST can be expressed and purified using the same protocol as the GST-PGP3 proteins.

- PGP3 derived from *C. trachomatis* pSW2 and pLGV440 expressed and purified as fusion proteins using the pGEX-6P-1 expression vector can be cleaved from the GST tag using Prescission Protease and are able to trimerise.
- Rabbit polyclonal anti-PGP3 antisera produced using recombinant cleaved PGP3 derived from *C. trachomatis* pSW2 can detect recombinant monomeric and trimeric GST-PGP3, recombinant PGP3 cleaved from the GST tag and PGP3 expressed in *C. trachomatis* L2 and *C. muridarum* Nigg elementary bodies.

CHAPTER FOUR: DEVELOPMENT AND OPTIMISATION OF INDIRECT ELISAS AND WESTERN BLOTS FOR THE DETECTION OF ANTI-PGP3 ANTIBODIES

4.1 INTRODUCTION

Measuring and understanding the seroprevalence of urogenital *C. trachomatis* infection in populations has been hindered by the lack of a sensitive and specific serological assay. Previous methods incorporating recombinant PGP3 in serological assays to detect anti-PGP3 antibodies in patient serum samples have been described (75, 118, 123, 160, 163, 167, 168). However, these methods vary in the type of serological assay used, the detection antigen, assay method and the number and type of controls used to optimise the assays, as summarised in Tables 1.4 and 1.5.

Most studies described previously have used indirect ELISAs to detect anti-PGP3 antibodies in patient serum samples (75, 118, 123, 160, 163). Indirect ELISAs involve coating plates in the detection antigen (PGP3) and the primary antibody (patient serum) is applied. Any antibodies bound to the recombinant PGP3 would then be detected by a labelled secondary antibody (e.g. anti-human IgG-HRP). Goodhew, *et al.* (2012) (167) incorporated the use of multiplex bead assays to detect anti-PGP3 antibodies in patient serum samples. This type of assay enables the detection of multiple analytes by coupling one or more antigens to polystyrene beads. Similar to an indirect ELISA, the beads are then incubated with human serum and any antibodies bound to antigens are detected by a labelled anti-human IgG secondary antibody. In this study, recombinant PGP3 and CT694 were used as detection antigens. However, the advantages of coupling antigens to beads prior to incubation with primary antibodies are unclear. The use of recombinant PGP3 using a sandwich ELISA method has also been undertaken. Li, *et al* (2013) (168) developed a sandwich ELISA using a monoclonal antibody to PGP3 and reported a high sensitivity and specificity of 92.1% and 100%, respectively. However, their motive for using a sandwich ELISA was to develop an assay for the serodiagnosis of *C. trachomatis* using urine or endocervical swabs. Therefore, similar to NAATs a sandwich ELISA would only be able to detect a current infection and would not be suitable for seroepidemiological studies. Taking the above considerations into account,

the decision was made to use indirect ELISAs incorporating recombinant PGP3 to detect anti-PGP3 antibodies in patient serum samples.

In the previous chapter, the expression and purification of recombinant PGP3 derived from the *C. trachomatis* plasmids pSW2 (serovar E) and pLGV440 (serovar L1) was described. These recombinant PGP3 proteins were expressed as fusion proteins with N-terminal polyhistidine or glutathione s-transferase (GST) tags to allow purification using affinity chromatography. This chapter describes the optimisation of ELISAs and Western blot assays to detect anti-PGP3 antibodies in patient serum samples as an indicator of prior exposure to *C. trachomatis*. The various recombinant PGP3 fusion proteins were also assessed for their ability to detect anti-PGP3 antibodies in human serum samples using indirect ELISAs and Western blot assay. Human sera were also assayed against his-tagged mouse norovirus 3C protease and glutathione s-transferase as negative controls to assess the possibility of false-positive reactions from human serum to the his- or GST- tags, respectively. In the previous chapter, the purification of PGP3 enzymatically cleaved from the GST tag using PreScission Protease was also described. However, this protein was not included in this chapter as it was only expressed and purified after the indirect ELISAs and Western blot assays were optimised and the final detection antigen was chosen, as explained further in chapter five. As previously discussed, it has been reported that the human antibody recognition of PGP3 is dependent on the native trimeric conformation of PGP3 (124, 125). As previous PGP3 ELISAs described the denaturing purification of recombinant PGP3 and the human antibody recognition of monomeric PGP3 (118, 160), the reactivity of human antibody recognition to monomeric and trimeric PGP3 was investigated further using Western blot assays.

4.1.1 Aim

- To produce a working ELISA for the detection of antibodies to *C. trachomatis* PGP3.

4.1.2 Objectives

- Determine the most effective blocking agent in reducing false background from human serum in indirect ELISAs and Western blots.

- Determine the most suitable method of background correction for the indirect ELISAs.
- Investigate the reactivity of human sera against all recombinant his-PGP3, GST-PGP3 and his-3C and GST negative control proteins in indirect ELISAs.
- Choose the optimal antigen concentration to use in indirect ELISAs.
- Investigate the effects of adding DTT and bicarbonate buffer [pH 9.6] to the trimeric structure of recombinant PGP3.
- Choose the optimal blocking agent, serum dilution and detection method for Western blots and investigate the potential reactivity of human sera to both monomeric and trimeric recombinant PGP3.

4.2 METHODS

4.2.1 Serum controls

4.2.1.1 Control antibodies

Positive control antibodies included rabbit polyclonal anti-PGP3 IgG, mouse monoclonal anti-GST antibody and mouse monoclonal anti-his antibody (Invitrogen, Life Technologies) (2.10.8.1 and 2.11.1).

4.2.1.2 Human serum samples

The human serum samples were supplied by PHE (NREC no. 05/Q0505/45) and the project is registered with Ethics and Research Governance Online (ERGO) at the University of Southampton. The sera were provided in three groups: (i) GUM male and female patients diagnosed as positive for *C. trachomatis* by NAAT; (ii) GUM patients diagnosed negative by NAAT; (iii) and non-GUM patients (assumed negative for *C. trachomatis*). The samples were anonymised and unlinked to preserve patient confidentiality.

4.2.2 Optimisation of indirect ELISAs

4.2.2.1 Assessment of blocking agents

5% non-fat milk (Marvel), 10% non-fat milk (Marvel), 3% BSA (Sigma-Aldrich), 1% sodium caseinate (Fisher Scientific) and 1% Hammarsten grade sodium caseinate (Affymetrix, eBioscience) blocking agents were assessed in their ability to prevent non-specific binding from human serum samples in an ELISA. This assessment was undertaken by coating Medisorp flat-bottom 96-well plates (Nunc, Thermo Scientific) in sodium bicarbonate buffer (2.11) at 100 µl/well for 2 hours at 37°C. Blocking agents in 0.1% PBS-Tween 20 (PBS-T) were added at 200 µl/well for 2 hours at 37°C. At every step, plates were washed six times in 0.1% PBS-T. Five randomly selected patient serum samples were added in duplicate at 1:100 (50µl/well) in 1% of the blocking buffers in PBS-T for 1 hour at 37°C. Goat anti-human IgG-HRP (BioRad) was added at 1:8000 and antibodies were detected as described (2.11).

4.2.2.2 Assessment of different preparations of Hammarsten-grade sodium caseinate blocking buffers

Hammarsten-grade sodium caseinate (Affymetrix, eBioscience) is purchased in a power form and must be treated to become soluble in solution to use as a blocking buffer in an ELISA. There are variations in the recommended temperatures and methods to dissolve the Hammarsten casein in solution (eBioscience personal communication). 1% Hammarsten-grade caseinate was prepared in 0.1% PBS-T by heating to 70°C and 100°C and used to block non-specific binding from human serum samples. Purified recombinant GST-PGP3 (3.3.3.1) derived from *C. trachomatis* pSW2, PGP3 (3.3.3.3) derived from *C. trachomatis* pSW2 and GST (3.3.3.2) were used to coat ELISA plates at 500 ng/well, 500 ng/well and 250 ng/well, respectively, as described (2.11). GST was used at 250 ng/well to match the fusion tag:polypeptide ratio of the GST-PGP3, as further explained in 4.2.2.4. Plates were washed in PBS-T and blocked using the prepared blocking buffers at 200µl/well for 2 hours at 37°C. Ten serum samples from patients with a known *C. trachomatis* status as diagnosed by NAAT and ten non-GUM samples (4.2.1.1) with a known reaction to GST-PGP3 or PGP3 in an ELISA were added in duplicate at 1:100 (50µl/well) in 1% of the prepared blocking buffers for 1 hour at 37°C. Plates were then washed, incubated with goat anti-human IgG-HRP at 1:8000 and antibodies were detected as described (2.11). The potential effects of the number of washes between steps in the ELISA were also investigated. The above experiment was repeated twice: once using three washes with PBS-T after every step, and again using six washes with PBS-T after every step.

4.2.2.3 Comparison of his- and GST-tagged PGP3 proteins as capture antigens

For the initial comparison of our antigens, the ELISA protocol described by Wills *et al.* was used as a guide (118). Medisorp flat-bottom 96-well plates (Nunc, Thermo Scientific) were coated for 2 hours at 37°C with purified recombinant antigen in carbonate-bicarbonate buffer [pH 9.6] at 100ng/well for the his-tagged PGP3 proteins and 200 ng/well for the GST-tagged PGP3 proteins to match the approximate fusion tag:recombinant polypeptide ratio, as described (2.11). MNV 3C (nS6) protease and glutathione s-transferase negative control antigens were added at 100 ng/well. At every step, plates were washed six times in 0.1% PBS-T. Plates were blocked using 1%

Hammarsten casein (4.2.2.2) in PBS-T at 200µl/well for 2 hours at 37°C. Patient sera were added at 1:100 (50µl/well) in 1% blocking buffer for 1 hour at 37°C. Positive and negative control serum samples and antibodies were selected as described in 4.2.1.1. Plates were incubated with goat anti-human IgG-HRP at 1:8000 and antibodies were detected as described (2.11). Background was corrected by subtracting the average absorbance of each duplicate well containing serum, blocking, conjugate and TMB but no antigen (uncoated wells), from the average absorbance of duplicate wells containing antigen (coated wells), serum, blocking, conjugate and TMB.

4.2.2.4 Capture antigen saturation

As described in 4.2.3.3, purified recombinant antigens in carbonate-bicarbonate buffer [pH 9.6] were used to coat 96-well plates at 100ng/well for the his-tagged PGP3 proteins and 200 ng/well for the GST-tagged PGP3 proteins to match the approximate fusion tag:recombinant polypeptide ratio. All new batches of proteins were titrated in an ELISA using 1-500 ng/well of antigen and samples of positive and negative control serum samples (4.2.1.1) and antibodies using the method described in 2.11. Background was corrected using the method described in 4.2.2.3.

4.2.2.5 Effects of antibody recognition of recombinant PGP3 containing DTT

Wills *et al.* (118) added 5 mM DTT to their recombinant PGP3 for use in an indirect ELISA. Although they state that adding DTT has no effect on the trimeric structure of PGP3, the reasons for adding it are not stated. We added 5 mM DTT to purified recombinant GST and GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 (3.3.3.3). Sera from patients diagnosed as positive for *C. trachomatis* by NAAT positive were then assayed against GST-PGP3 and GST antigens with and without added 5 mM DTT in an ELISA (2.11). Five negative control serum samples used as negative controls were also assayed (4.2.1.1). Mouse monoclonal anti-GST IgG (1:500) and rabbit polyclonal anti-PGP3 IgG (1:100) antibodies were used as positive controls. Antigens were used to coat Medisorp flat-bottom 96-well plates at 500 ng/well (GST: 250 ng/well) in carbonate-bicarbonate buffer [pH 9.6] for 2 hours at 37°C, as described (2.11). Plates were washed three times in 0.1% PBS-T. Plates were blocked using 1% Hammarsten casein in PBS-T at 200µl/well for 2 hours at 37°C. Sera and secondary antibodies were added and antibodies were detected as described (2.11). Background was corrected as described in 4.2.3.3.

4.2.2.6 Effects of adding sodium bicarbonate buffer [pH 9.6] to the trimeric structure of recombinant PGP3

To investigate the potential effects of adding sodium bicarbonate buffer, an alkaline solution, to the native trimeric structure of recombinant PGP3, we added 5 mM sodium bicarbonate coating buffer [pH 9.6] (2.11) to purified recombinant GST-PGP3 (3.3.3.1) and PGP3 (3.3.3.3) derived from *C. trachomatis* pSW2, and GST (3.3.3.2). Purified recombinant proteins were diluted to 5 µg/ml in 5 mM sodium bicarbonate buffer [pH 9.6] for 2 hours at 37°C (to mimic the 500 ng/well antigen concentration, as described in 4.2.2.4). As a control, proteins diluted in sodium bicarbonate buffer were compared with protein diluted to 5 µg/ml in PBS for 2 hours at 37°C. 30 µl aliquots were added to 10 µl 4x native loading buffer (no SDS or BME, 2.10.2), analysed by SDS-PAGE on 12.5% SDS gels (2.10) and transferred to a PVDF membrane (2.10.7). Membranes were blocked as described (2.10.8) and incubated with rabbit polyclonal anti-PGP3 IgG antisera (1:100) (derived from *C. trachomatis* pSW2, produced by INC group, 3.2.3) for 1 hour at room temperature. The membrane was washed and antibodies were detected using an ECL kit, as described (2.10.8.3).

4.2.3 Optimisation of Western blots

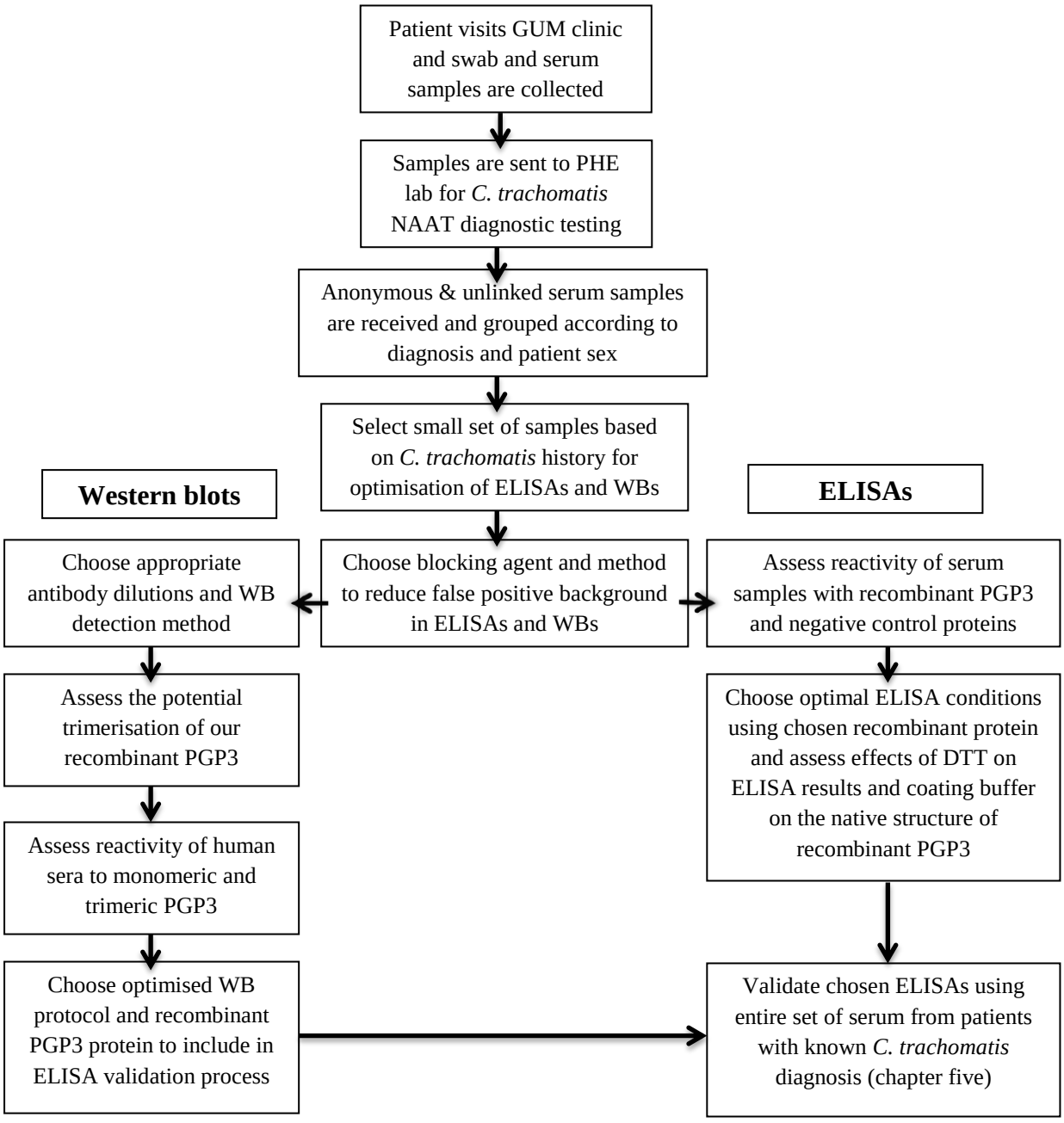
4.2.3.1 Assessment of blocking agents, antibody dilution and detection

1% Hammarsten grade casein, 5% BSA and 5% skimmed milk solution in 0.1% PBS-T were assessed in their ability to reduce false-positive background from human serum samples in a Western blot. 30 µl aliquots of recombinant GST-PGP3 (3.3.3.1) from *C. trachomatis* pSW2 and GST were denatured in 10 µl 4x SDS loading buffer (2.10.2), 10 µl aliquots were analysed by SDS-PAGE (2.10) and transferred to a PVDF membrane, as described (2.10.7). Human serum samples selected based on their *C. trachomatis* status as diagnosed by NAAT (4.2.1.1) were added to the membranes at 1:100 dilution in 1% of the blocking buffers in 0.1% PBS-T. Antibodies were then detected as described (2.10.8) using an ECL kit or the colorimetric system. Additionally, the optimal primary antibody and serum dilution, membrane and X-ray exposure time and the most effective method of detection (ECL, colorimetric) were also investigated.

4.2.3.2 Antibody recognition of monomeric and trimeric recombinant PGP3

The potential antibody recognition of both monomeric and trimeric recombinant PGP3 from human antibodies was investigated. 10 µl aliquots of recombinant GST-PGP3 (3.3.3.1) derived from *C. trachomatis* pSW2 were denatured or left untreated (2.10.2) to view monomeric and trimeric GST-PGP3, respectively, and were analysed by SDS-PAGE on 12.5% gels. GST was also included as a negative control to assess potential non-specific reaction from human sera to the GST tag. Proteins were then transferred to a PVDF membrane (2.10.8) and blocked in 10% skimmed milk solution (2.10.8). 12 serum samples from patients diagnosed as positive for *C. trachomatis* by NAAT, 10 serum samples from patients diagnosed negative and 5 samples from non-GUM patients were added at 1:100 and 1:2000 dilutions to the membranes. Membranes were incubated and antibodies detected as described (2.10.8).

Figure 4.1. Strategy for the optimisation of ELISAs and Western blotting experiments using human serum samples. Flowchart also shows the chronological order at which experiments were conducted.



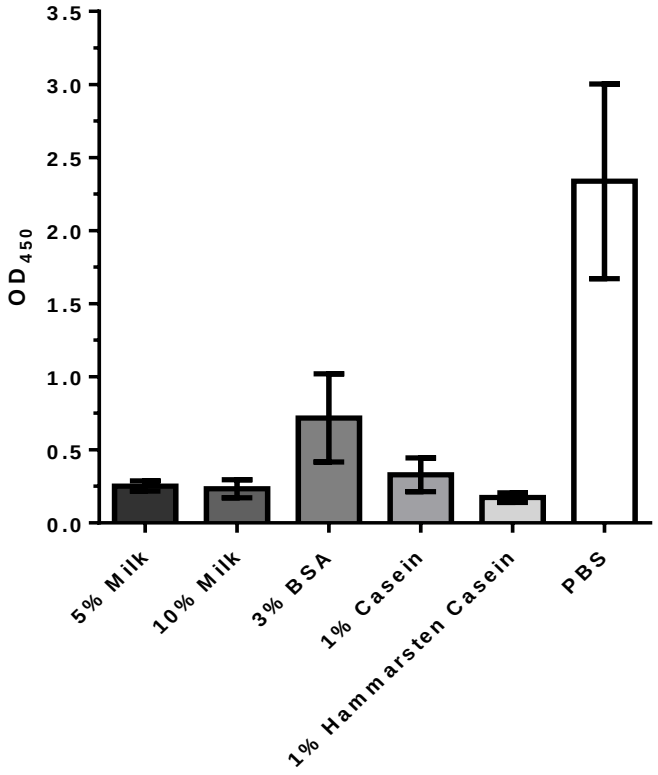
4.3 RESULTS

4.3.1 Assessment of blocking agents in reducing non-specific background in indirect ELISAs

Many problems due to false positive background from human serum samples in ELISAs were encountered in the initial experiments. Initial problems in preventing false-positive signals in the ELISA were due to insufficient blocking of the wells and non-specific binding of products in the serum samples. Various experiments were then undertaken to determine the most appropriate blocking agent to prevent this non-specific binding (4.2.2.1). 5% non-fat milk, 10% non-fat milk, 3% BSA, 1% sodium caseinate and 1% Hammarsten-grade sodium caseinate were assessed as candidate blocking agents for an ELISA using five randomly-selected serum samples in uncoated wells against a PBS control (unblocked wells) (4.2.2.1). Serum samples were assayed at 1:100 and antibodies were detected using goat anti-IgG-HRP at 1:8000. These antibody dilutions were used as an initial guide, according to the assay described by Wills *et al*. (118, 184). The mean OD_{450} of the sera in the unblocked (PBS) well controls was 2.33. Statistical analysis (2.12) was conducted using one-way ANOVA (P value for all blocking agents vs control (PBS/unblocked) = <0.0001). Tukey multiple comparisons test of 1% Hammarsten-grade sodium caseinate and 10% skimmed milk OD_{450} values revealed no significant differences. However, 1% Hammarsten casein was the most appropriate blocking agent in reducing false-positive signals as this blocking agent produced the lowest OD values in comparison to the other blocking agents (mean OD_{450}: 0.173). 3% BSA was the least effective in blocking non-specific binding as this blocking agent produced the highest OD values (OD_{450}: 0.718). This comparison of the blocking agents can be seen in Figure 4.2 (raw OD_{450} values are displayed).

Figure 4.2. Assessment of blocking agents in reducing false-positive background in an ELISA using patient serum samples.

Uncoated wells were blocked with 5% and 10% non-fat milk, 3% BSA, 1% sodium caseinate or 1% Hammarsten grade sodium caseinate for 2 hours at 37°C. Five randomly-selected human serum samples were assayed at 1:100 in 1% of the blocking buffers and incubated with goat anti-human IgG-HRP (1:8000). Sera were assayed in unblocked wells (PBS) as a control. Five serum samples were assayed in duplicate to assess the blocking agents and statistical analysis was conducted using one-way ANOVA and mean with standard deviation (raw OD_{450} (non-background corrected) values are displayed). [P value for all blocking agents vs control (PBS/unblocked) = <0.0001].



4.3.2 Method of background correction in indirect ELISAs

Due to the outcomes of the above experiment (4.3.1) and other initial ELISA optimisation experiments it became apparent that each serum sample used in uncoated ELISA wells produced individual and very different absorbance readings. 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 (4.2.2.3).

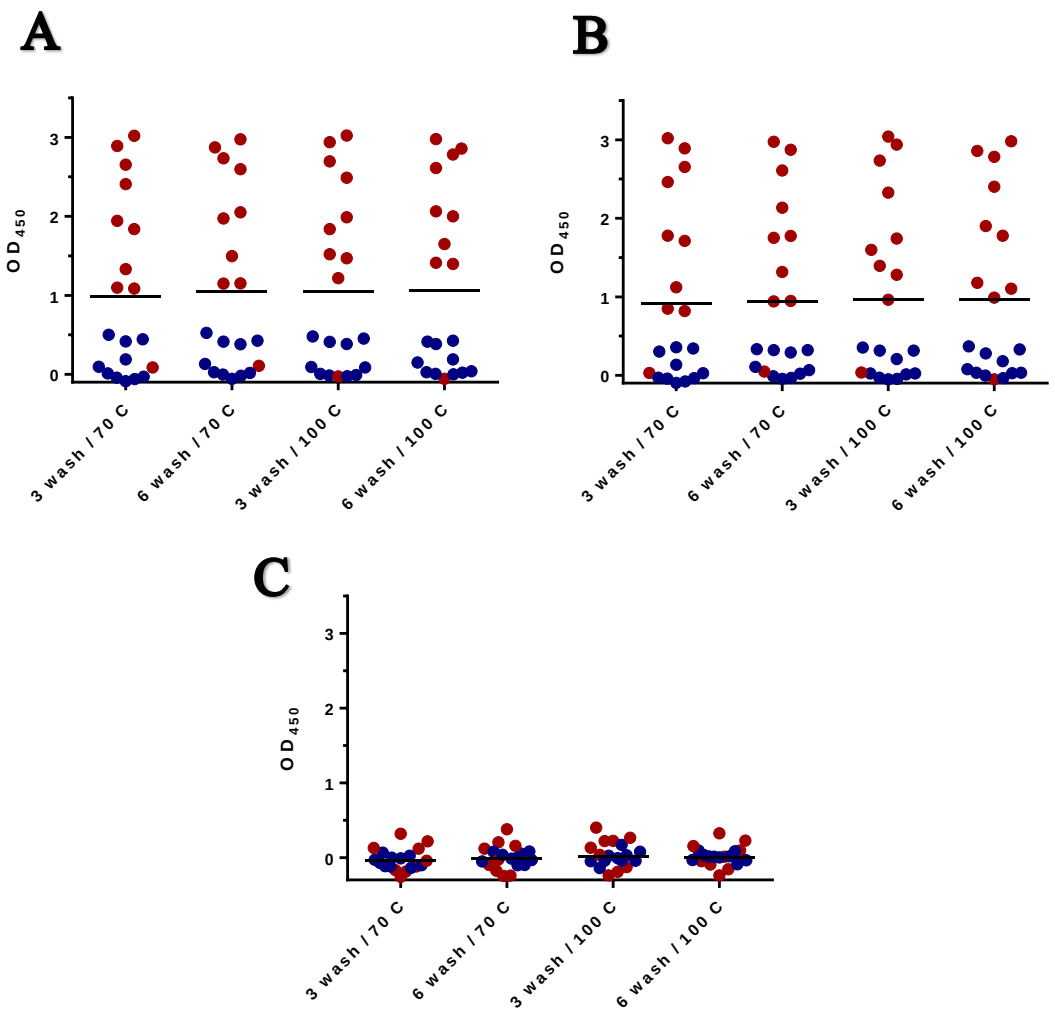
4.3.3 Preparation of Hammarsten-grade casein and effect of the number of washes on ELISA results

Hammarsten grade sodium caseinate from Affymetrix (eBioscience) is purchased in a powder form and requires heating to allow the casein to dissolve into solution. There are two common methods to improve the solubility of casein. These include dissolving the casein in 1M NaOH overnight and then adjusting to the preferred pH or by heating the casein in solution until fully dissolved (eBioscience communication). As we wanted to preserve the potential trimeric structure of our recombinant PGP3 within the ELISA, and for a faster preparation, we chose to heat our casein blocking buffer. However, there are variations in the recommended temperatures to use to dissolve the Hammarsten casein in solution (eBioscience communication)**.** All materials for our PGP3 ELISAs (PBS tablets, Tween 20, HRP conjugates, ready-made TMB solution and H_2SO_4) are purchased from various manufacturers and are less likely to have an effect on inter-operator or inter-assay variation between ELISAs. However, as each batch of Hammarsten casein blocking buffer is prepared individually for each assay there is a possibility of slight temperature differences used for the preparation of blocking buffer which may result in different levels of solubilised casein. The resulting blocking buffer would then be used to block unbound sites in the ELISA wells and then be used for serum and conjugate antibody dilution preparations. Collectively, these may result in differences between OD_{450} values on the same samples between different independent assays. We therefore wanted to investigate if the same blocking agent prepared using different methods would result in different background readings in an ELISA. We conducted an ELISA (2.11) using recombinant GST-PGP3 (3.3.3.1) derived from *C. trachomatis* pSW2, cleaved PGP3 (3.3.3.3) derived from *C. trachomatis* pSW2 and GST as a negative control (3.3.3.2), and blocked ELISA plates using Hammarsten

casein prepared by heating to 70°C (partially dissolved) or to 100°C (fully dissolved). Ten patient serum samples (4.2.1.1) known to give a range of high, medium and low OD_{450} values when assayed against GST-PGP3 SW2 (500 ng/well) in an ELISA were selected. Ten non-GUM patient serum samples were also selected as negative controls. These sera were also assayed against cleaved PGP3 SW2 (500 ng/well) and the GST negative control antigen (250 ng/well). Additionally, to investigate the potential effect of the number of washes with PBS-T after various steps in the ELISA, we repeated the above experiment twice: once with three washes after each step and the other with six washes. Sera were assayed at 1:100 and antibodies were detected using goat anti-human IgG-HRP (1:8000). For all antigens, no statistical significance was found between OD_{450} values obtained from plates that had undergone six washes and those that had undergone three washes (Tukey multiple comparisons test (2.12); all p: >0.05). Additionally, no statistical significance was found between OD_{450} values obtained from plates that had been blocked with Hammarsten casein blocking reagent prepared to 70°C or 100°C (Tukey multiple comparison test; all p: >0.05). All statistical tests were calculated separately for positive and negative control sera. Background corrected OD_{450} values of the negative non-GUM sera ranged from OD_{450} -0.086-0.520 against GST-PGP3 SW2 and OD_{450} -0.094-0.37 against PGP3 SW2 across all plates with all prepared blocking reagents that had undergone 3 or 6 washes at each step. OD_{450} values of the sera from patients diagnosed positive for *C. trachomatis* by NAAT ranged from OD_{450} -0.056-3.26 against GST-PGP3 SW2 and OD_{450} -0.052-3.05 against PGP3 SW2 across all assay conditions. OD_{450} values of all sera assayed against GST negative control antigen ranged from OD_{450} -0.256-0.38 across all assay conditions. Comparison of these OD_{450} values can be seen in Figure 4.3.

Figure 4.3. Assessment of different preparations of Hammarsten-grade casein as a blocking agent and effects of the number of washes on indirect ELISA results.

Graph shows mean background-corrected OD_{450} values of 10 serum samples from GUM patients (red points) and 10 serum samples from non-GUM patients (blue points) with a previous known reaction to recombinant GST-PGP3 and PGP3 in an ELISA. GST-PGP3 SW2 and PGP3 SW2 were used to coat plates at 500 ng/well and GST at 250 ng/well in sodium bicarbonate buffer [pH 9.6]. Plates were blocked in 1% Hammarsten-grade casein prepared by heating to 70°C or 100°C and sera were assayed in duplicate at 1:100. Antibodies were detected using goat anti-human IgG-HRP (1:8000). Plates were washes 3 or 6 times at every step. Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean bars are shown. **[A]** GST-PGP3 SW2; **[B]** PGP3 SW2; **[C]** GST (negative control).



4.3.4 Comparison of recombinant his- and GST-tagged PGP3 proteins as capture antigens to detect anti-PGP3 antibodies in human serum an indirect ELISA

4.3.4.1 His-tagged PGP3 derived from C. trachomatis pSW2 and pLGV440

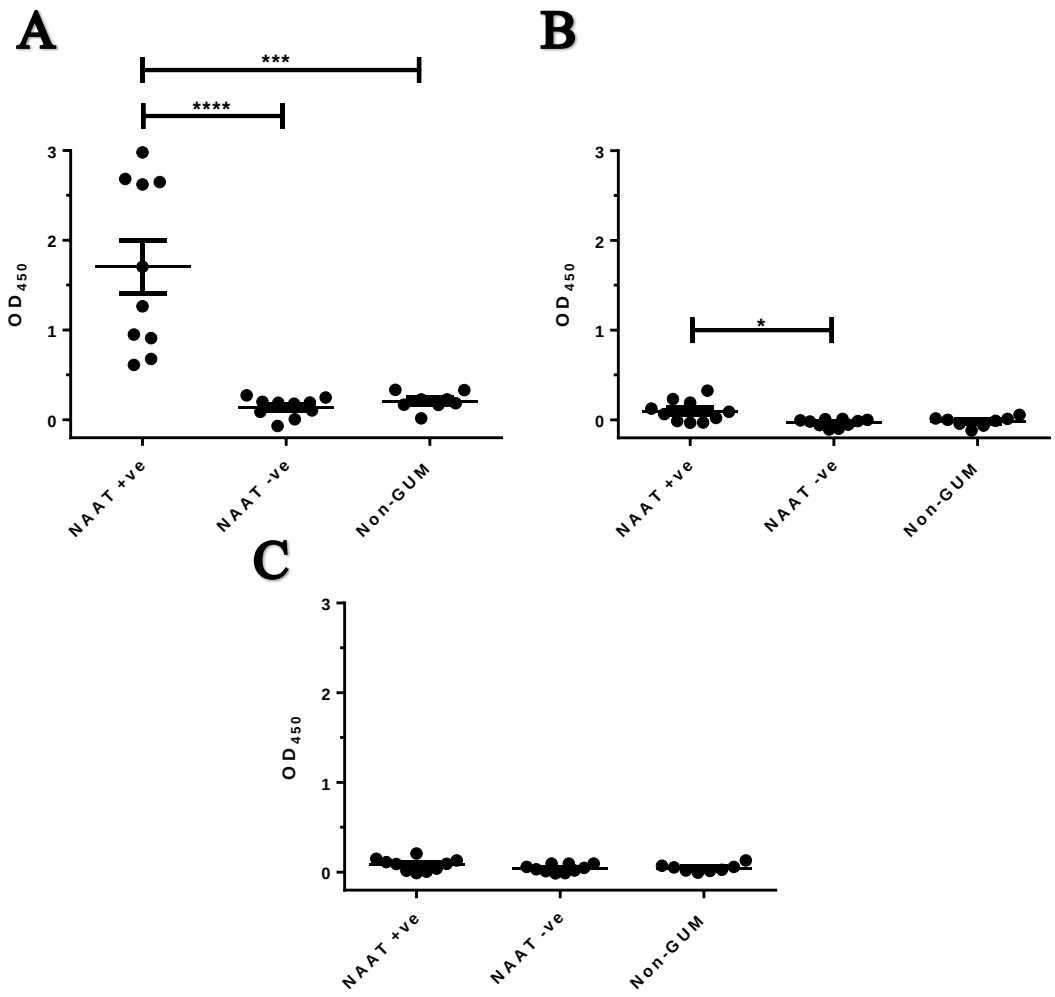
Using an indirect ELISA and serum samples from patients with a known *C. trachomatis* diagnosis (by NAAT), we assessed the potential of our recombinant his-tagged PGP3 proteins (3.3.2.1) to detect/bind anti-PGP3 antibodies in human sera. Recombinant his-tagged PGP3 derived from both *C. trachomatis* pSW2 and pLGV440 (3.3.2.1) were used to perform an ELISA (2.11) using 10 sera from patients diagnosed positive for *C. trachomatis* by NAAT, 10 sera from patients diagnosed negative for *C. trachomatis* by NAAT and 8 negative control sera from a non-GUM source (NAAT status unknown) (4.2.1.1). During ELISA optimisation, the Wills *et al.* PGP3 ELISA was used as an initial guide to test the reactivity of human sera with our recombinant PGP3 antigens and so plates were coated with 100 ng/well antigen (2.11). Sera were assayed at 1:100 in duplicate, as described (4.2.2.3). The OD_{450} values were corrected for background (4.2.2.3) and grouped according to their NAAT status (Figure 4.4). One-way ANOVA with Tukey multiple comparisons tests were used to compare the groups and calculate the means and standards deviations (2.12). In both his-PGP3 SW2 and LGV440 ELISAs, no statistical significances were found between sera from patients diagnosed negative for *C. trachomatis* by NAAT and the non-GUM sera ($p = >0.05$). The background-corrected OD_{450} values in both groups ranged from -0.155 to 0.411 in both his-tagged PGP3 SW2 and LGV440 ELISAs. For the his-PGP3 SW2 ELISA, statistical significance was found between the sera from patients positive for *C. trachomatis by* NAAT and the sera from patients diagnosed negative ($p = <0.0001$). Statistical significance was also found between the sera from GUM patients diagnosed positive for *C. trachomatis* by NAAT and the non-GUM sera ($p = 0.0004$). The OD_{450} values of the sera positive for *C. trachomatis* by NAAT ranged from 0.612 to 2.97. In the his-PGP3 LGV440 ELISA, although also significant, less statistical significance was found between the sera from patients diagnosed positive for *C. trachomatis* by NAAT and the sera from patients diagnosed negative ($p = 0.004$). No statistical significance was found between the non-GUM sera and sera from GUM patients diagnosed negative for *C. trachomatis* by NAAT or sera from patients diagnosed positive ($p = >0.05$). In contrast to the his-PGP3 SW2 ELISA, for the his-PGP3 LGV440 ELISA the OD_{450} values of the sera positive for *C. trachomatis* by NAAT had a lower range between -0.027 to 0.326.

4.3.4.2 His-3C (ns6) protease

Purified mouse norovirus his-tagged 3C (nS6) protease (3.3.2.2) was also used to perform an ELISA using the above described positive and negative control patient sera (4.3.3.1). The 3C (nS6) protease was chosen as a negative control protein for the his-tagged PGP3 ELISAs as it was able to be expressed and purified using the same protocol for the his-PGP3 derived from *C. trachomatis* pLGV440, as described in 3.2.2.1. This negative control protein was also expressed with an N-terminal histidine tag and was used to eliminate any possibility of false positive values from reactivity of the sera with the his-tag in an ELISA. The mouse norovirus protein was also chosen to assess the non-specific binding of components in the sera to the antigen that would produce false positive results. Since this particular 3C (nS6) protease was derived from mouse norovirus, no reaction from human sera in an ELISA is expected. The sera described above (4.3.3.1) were assayed at 1:100 in duplicate against his-3C in an indirect ELISA as described (4.2.2.3). OD_{450} values were corrected for background and grouped according to their NAAT status (Figure 4.4). One-way ANOVA with Tukey multiple comparisons tests (2.12) were used to compare the groups and calculate the means and standards errors. No statistical significance was found between all of the groups in the 3C (nS6) protease ELISA. Background-corrected OD_{450} values ranged between -0.033 to 0.208 across all serum groups. Statistical significance was found between all serum groups in the 3C (nS6) protease ELISA and the sera from patients diagnosed positive for *C. trachomatis* by NAAT in both the his-PGP3 SW2 (all $p = <0.0001$) and his-PGP3 LGV440 ELISAs (all $p = 0.003$-0.007). No statistical significance was found between the non-GUM sera and sera from patients diagnosed negative for *C. trachomatis* by NAAT in the his-PGP3 ELISAs and all the groups in the 3C (nS6) protease ELISA (all $p = >0.05$). Two-way ANOVA (2.12) was used to calculate statistical significances between different serum groups assayed against different antigens.

Figure 4.4. Antibody responses from human serum assayed against recombinant his-tagged PGP3 derived from *C. trachomatis* pSW2 or pLGV440 and his-tagged MNV 3C (nS6) protease in an indirect ELISA.

This graph shows the background-corrected OD_{450} of sera from GUM patients known to be positive or negative for *C. trachomatis* by NAAT and sera from non-GUM patients (NAAT status unknown). Recombinant his-PGP3 and his-3C at 100 ng/well in sodium bicarbonate buffer [pH 9.6] were used to coat plates. Sera were assessed in duplicate at 1:100 and antibodies detected using goat anti-human IgG-HRP (1:8000). Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean and standard deviation bars are shown [One-way ANOVA and Tukey multiple comparison test: ****: p = <0.0001; ***: p = <0.001; **: p = <0.01; *: p = <0.05]. **[A]** His-PGP3 SW2; **[B]** His-PGP3 LGV440; **[C]** Mouse norovirus his-3C (nS6) protease (negative control).



4.3.4.3 GST-tagged PGP3 from C. trachomatis pSW2 and pLGV440

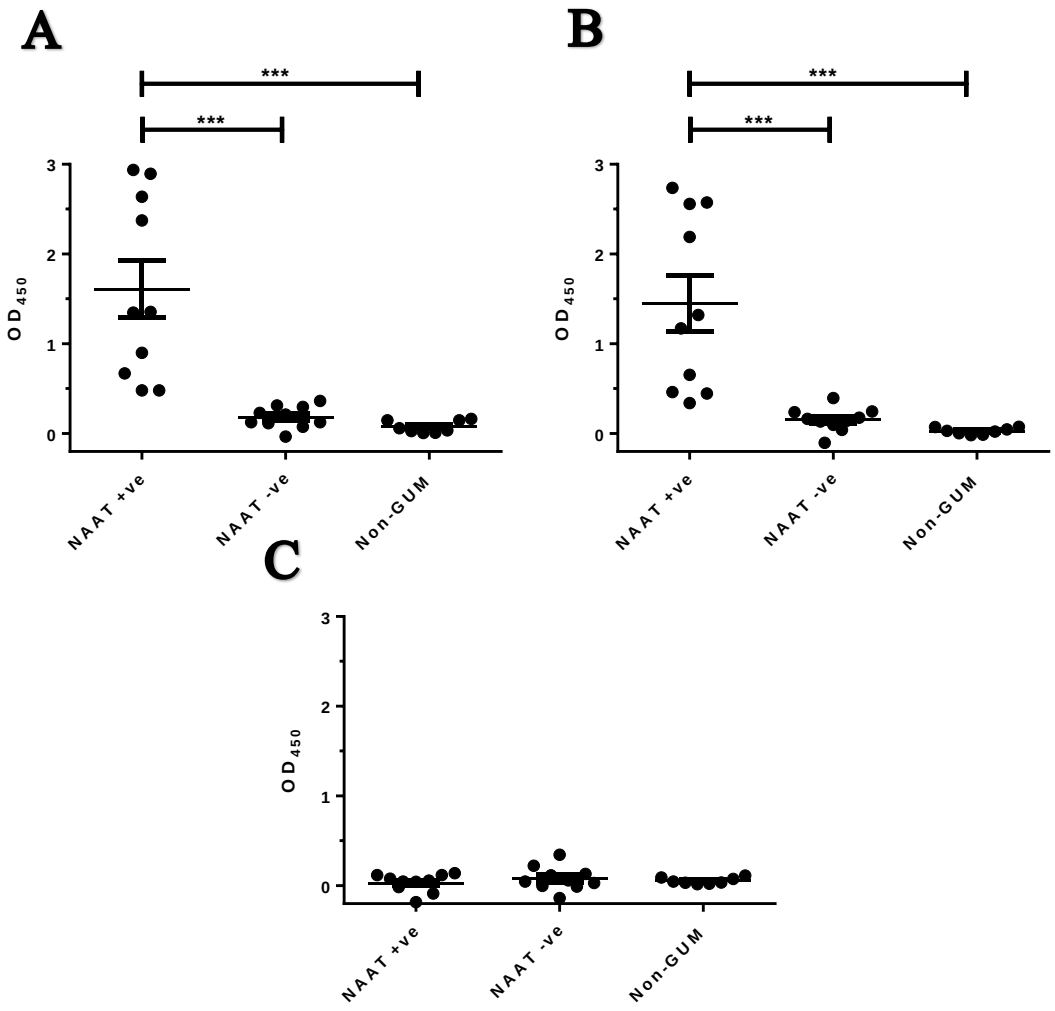
Using an indirect ELISA and serum samples from patients with a known *C. trachomatis* diagnosis (by NAAT), we assessed the potential of our recombinant GST-tagged PGP3 proteins (3.3.3.1) to detect anti-PGP3 antibodies in patient sera. Purified recombinant GST-tagged PGP3 derived from *C. trachomatis* pSW2 and pLGV440 (3.3.3.1) were used to perform an ELISA using 10 sera from patients diagnosed positive for *C. trachomatis* by NAAT, 10 sera from patients diagnosed negative and 8 sera from a non-GUM source (NAAT status unknown) (4.2.1.1). As described in 4.2.2.3, the Wills *et al.*, (2009) PGP3 ELISA was used as an initial guide and plates were coated in 100 ng/well of his-PGP3 antigen for the indirect ELISA (2.11). However, to match the tag:polypeptide ratio used by the his-PGP3 ELISAs, plates were coated in 200 ng/well GST-PGP3 antigen. Sera were assayed at 1:100 in duplicate, as described (4.2.2.3). The OD_{450} values were corrected for background and grouped according to their NAAT status (Figure 4.5) (4.2.2.3). One-way ANOVA with Tukey multiple comparisons tests were used to compare the groups and calculate the means and standards errors (2.12). No statistical significances were found between the sera from patients diagnosed negative for *C. trachomatis* by NAAT and the non-GUM sera groups (all $p = >0.05$). The OD_{450} values in both groups ranged from -0.034 to 0.362 in both GST-tagged PGP3 SW2 and LGV440 ELISAs. Statistical significance was found between the sera from patients diagnosed positive for *C. trachomatis* by NAAT and sera from patients diagnosed negative ($p = 0.0003$) and non-GUM sera ($p = 0.0006$) in the GST-PGP3 SW2 ELISA. The OD_{450} values of the sera from patients diagnosed positive for *C. trachomatis* by NAAT ranged from 0.481 to 2.93. In contrast to the his-PGP3 LGV440 ELISA, there was higher statistical significance in the GST-PGP3 LGV440 ELISA between the sera from patients diagnosed positive for *C. trachomatis* by NAAT and both the sera from patients diagnosed negative ($p = 0.0006$) and non-GUM sera ($p = 0.0009$). The OD_{450} values of the sera positive for *C. trachomatis* by NAAT ranged from 0.339 to 2.73 in the GST-PGP3 LGV440 ELISA. Note that these human sera were not assayed against recombinant cleaved PGP3 (3.3.3.3) as this protein was only expressed and purified after GST-PGP3 was chosen for the final assay validation (for further details, see chapter 5).

4.3.4.4 Glutathione s-transferase

Glutathione s-transferase (GST) was used to perform an ELISA using the above described GUM and non-GUM patient sera (4.3.3.3). To match the fusion tag:polypeptide ratio of GST-PGP3, GST was used to coat plates at 250 ng/well and sera were assayed at 1:100 in duplicate, as described (4.2.2.3). GST was chosen as a negative control for the recombinant GST-tagged PGP3 proteins to observe any false positive reaction from the human sera with the GST tag. Recombinant GST purified from the pGEX expression vectors (GE Healthcare) are derived from *Schistosoma japonicum*, one of the main causative agents of schistosomasis in rural regions of Asia, particularly China (185). Therefore, any sera collected from England will be unlikely to react with GST. OD_{450} values were corrected for background and grouped according to their patient NAAT status (Figure 4.5) (4.2.2.3). No statistical significance was observed between any of the groups of sera in the GST ELISA (all $p = >0.05$). OD_{450} values ranged between -0.184 to 0.346 across all groups. Furthermore, no statistical significance was found between any of the groups in the GST ELISA and the non-GUM and sera from patients diagnosed negative for *C. trachomatis* by NAAT in both the GST-PGP3 SW2 and LGV440 ELISAs ($p = >0.05$). Statistical significance was found between sera from patients diagnosed positive for *C. trachomatis* by NAAT in both GST-PGP3 SW2 ($p = 0.0001$) and LGV440 ELISAs ($p = 0.0003$) and the corresponding groups in the GST ELISA. Two-way ANOVA was used to calculate statistical significances between different serum groups assayed against different antigens (2.12).

Figure 4.5. Antibody responses from human serum assayed against recombinant GST and GST-tagged PGP3 derived from *C. trachomatis* pSW2 or pLGV440 in an indirect ELISA .

Graph shows the background-corrected OD_{450} of sera from GUM patients known to be positive or negative for *C. trachomatis* by NAAT and sera from non-GUM patients (NAAT status unknown). Recombinant GST-PGP3 (200 ng/well) and GST (100 ng/well) in sodium bicarbonate buffer [pH 9.6] were used to coat plates. Sera were assessed in duplicate at 1:100 and antibodies detected using goat anti-human IgG-HRP (1:8000). Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean and standard deviation bars are shown [One-way ANOVA and Tukey multiple comparison test: ****: p = <0.0001; ***: p = <0.001; **: p = <0.01; *: p = <0.05]. **[A]** GST-PGP3 SW2; **[B]** GST-PGP3 LGV440; **[C]** GST (negative control).



4.3.5 Capture antigen saturation

The initial antigen coating concentrations of 100 ng/well (his-PGP3) and 200 ng/well (GST-PGP3) for our ELISAs were chosen as a guide using the ELISA described by Wills *et al.* (118). These antigen concentrations were chosen for the preliminary experiments with the different recombinant PGP3 antigen formats, described in 4.2.2.3. However, during the production of new batches of proteins, it was discovered that the antigen concentrations used previously were not at saturation in the assay. It was found that even minor differences in the antigen coating concentration produced differences in the OD_{450} results in both anti-PGP3 antibody positive and negative sera. We thus conducted several titration experiments on subsequent batches of proteins using positive controls including mouse monoclonal anti-GST IgG antibody (1:500-10,000) and rabbit polyclonal anti-PGP3 IgG (1:100-10,000) (derived from *C. trachomatis* pLGV440 or pSW2 CDS5). Several sera collected from non-GUM patients were selected as negative controls and sera from GUM patients diagnosed as positive to *C. trachomatis* by NAAT were selected as positive controls (4.2.1.1). Sera were assayed in duplicate at 1:100 against antigens of increasing concentrations from 0-500 ng/well. All antigens assayed approach saturation at 300 ng/well and become fully saturated by 500 ng/well. As a result, all new assays conducted used an antigen coating concentration of 500 ng/well to ensure saturation of the assay. To match the fusion tag:polypeptide ratio of the GST-PGP3 proteins, GST was used at a concentration of 250 ng/well, although positive control antibodies revealed GST assay saturation at a lower concentration. As part of our 'in-house' test for all new batches of recombinant protein**,** titration assays were conducted to determine their point of saturation. Examples of ELISA plate saturation test can be seen in Figure 4.6. In this experiment, the positive control antibodies described above, four serum samples from non-GUM patients and four serum samples from GUM patients diagnosed positive for *C. trachomatis* by NAAT were assayed at 1:100 in duplicate against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST negative control antigen in an ELISA (2.11).

4.3.5.1 GST-PGP3 SW2

In the GST-PGP3 SW2 assay saturation test (Figure 4.6A), the antigen approaches the saturation point between 250-300 ng/well and OD_{450} values across all sera increase no more than 0.3 up to 500 ng/well. Two-way ANOVA and Tukey multiple comparisons tests (2.12) were used to calculate significant differences between mean background-

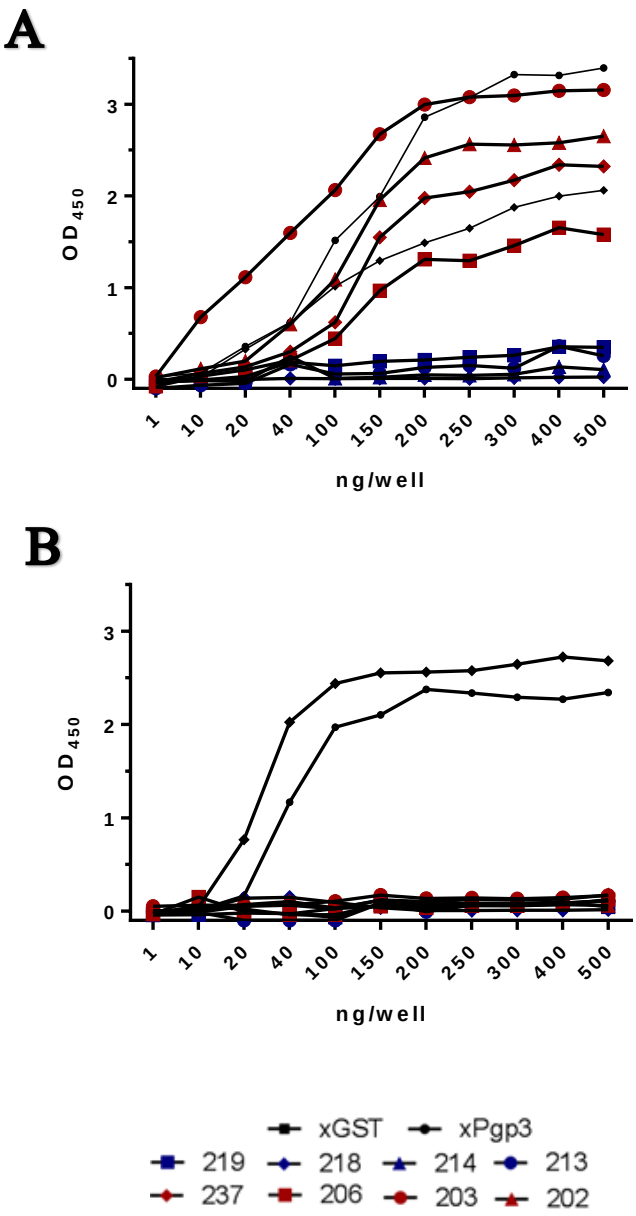
corrected OD_{450} values obtained from sera assayed against different concentrations of antigen. Data from positive control sera and negative control sera were calculated separately. Comparison of OD_{450} values obtained from sera from patients diagnosed positive for *C. trachomatis* by NAAT assayed against increasing concentrations of antigen found statistical significance until 250 ng/well of antigen (p value ranges = 0.001-0.05). Comparison of OD_{450} values obtained from the same sera against 300-500 ng/well found no statistical significances (all p values = >0.05). Anti-GST and anti-PGP3 positive control antibodies were included in these analyses. OD_{450} ranges of sera from patients diagnosed positive for *C. trachomatis* by NAAT across all antigen concentrations ranged between -0.0775-3.15. Comparison of OD_{450} values obtained from non-GUM sera assayed against 1-20 ng/well antigen with concentrations over 400 ng/well found some significant differences (all p values = 0.0001-0.05). No further significant differences were found when comparing OD_{450} values obtained from over 20-500 ng/well antigen (all p values =>0.05). OD_{450} ranges of non-GUM sera across all antigen concentrations ranged between -0.128-0.349.

4.3.5.2 GST

OD_{450} values in the GST negative control assay saturation test (Figure 4.6B) obtained from both non-GUM sera and sera from patients diagnosed as positive for *C. trachomatis* by NAAT remained low across all antigen concentrations (OD_{450} range -0.198-0.168). Some significant differences between OD_{450} values obtained from both non-GUM and NAAT positive sera were found when comparing 1-100 vs 150-500 ng/well antigen (p value of all comparisons = <0.05). However, comparison of OD_{450} values obtained from 150-500 ng/well antigen found no further significant differences (all p values = >0.05). The OD_{450} values from the mouse monoclonal anti-GST (1:500) and rabbit polyclonal anti-PGP3 positive control antibodies (1:10,000) approach the assay saturation point at 150-200 ng/well. OD_{450} values of both antibodies increase no higher than OD_{450} 0.2 from 250 to 500 ng/well of antigen.

Figure 4.6. Determination of saturation points of recombinant GST and GST-PGP3 derived from *C. trachomatis* pSW2 using patient sera in an indirect ELISA.

The graph shows the background-corrected OD_{450} of four sera known to be positive for *C. trachomatis* by NAAT and four negative control non-GUM sera (NAAT status unknown) assayed against increasing concentrations of recombinant GST and GST-PGP3 derived from *C. trachomatis* pSW2. Antigen was added between 0-500 ng/well in sodium bicarbonate buffer [pH 9.6]. Sera were assessed in duplicate at 1:100 and antibodies detected using goat anti-human IgG-HRP (1:8000). Red points are sera from GUM patients and blue points are sera from non-GUM patients. Mouse monoclonal anti-GST (1:10,000) and rabbit polyclonal anti-PGP3 (1:10,000) were included as positive controls. Each point shows the mean absorbance of the two duplicate values. **[A]** GST-PGP3 SW2; **[B]** GST

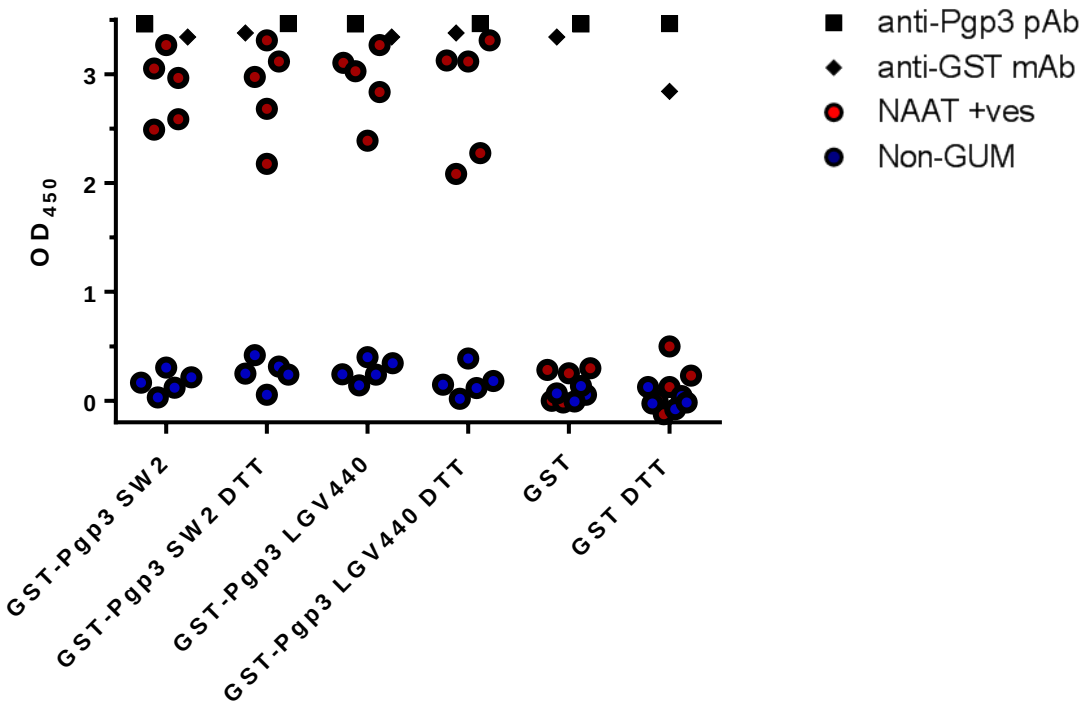


4.3.6 Effects of antibody recognition of recombinant PGP3 containing Dithiothreitol

During the purification of recombinant PGP3 derived from *C. trachomatis* pLGV440, Wills *et al.* (2009) added 5 mM dithiothreitol (DTT) to their purified protein fractions. However, although they state that DTT does not affect their ELISA, they do not explain their reasons for adding DTT. Since DTT is known to reduce disulphide bonds which may result in protein structural changes, and our previous Western blot experiments revealed some patient sera reacting with only the PGP3 trimer (see 4.3.8), we sought to investigate the effects of adding DTT to our recombinant GST-PGP3 antigens and observe any potential differences in OD_{450} values in an ELISA. 500 ng/well of GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 (3.3.3.1) (with and without added 5 mM DTT) were used to perform an ELISA (2.11) using sera from five GUM patients diagnosed as positive for *C. trachomatis* by NAAT (4.2.2.1). Five sera from a non-GUM source were also used as negative controls. All sera were assayed at 1:100 in duplicate against GST-PGP3 SW2 and GST-PGP3 LGV440 antigens and OD_{450} values were corrected for background. Mean absorbances were calculated and grouped according to the antigen and added DTT. One-way ANOVA with multiple comparisons tests (2.12) were used to compare the groups and calculate means (Figure 4.7). Although some minor differences in OD_{450} values were observed between antigens with and without added DTT, no statistical differences were observed between OD_{450} values obtained from sera assayed against antigen with DTT and those assayed against antigen without DTT. The largest difference in OD_{450} values between sera assayed was a decrease from OD_{450} 2.491 to 2.176 between GST-PGP3 SW2 without DTT and with added DTT, respectively. There was also a decrease in OD_{450} value from 3.34 to 2.84 using the mouse monoclonal anti-GST IgG antibody against GST and GST with added DTT, respectively. No differences in OD_{450} were observed using the rabbit polyclonal anti-PGP3 IgG against antigens with and without added DTT.

Figure 4.7. Effects of antibody recognition of recombinant PGP3 containing Dithiothreitol.

Recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (500 ng/well), GST-PGP3 derived from *C. trachomatis* pLGV440 (500 ng/well) and GST (250 ng/well) in sodium bicarbonate buffer [pH 9.6] were used to coat plates, with or without added 5 mM DTT, and sera were assessed in duplicate at 1:100. Antibodies were detected using goat anti-human IgG-HRP (1:8000). Sera included five from GUM patients diagnosed as positive for *C. trachomatis* by NAAT and five sera from a non-GUM source. Mouse monoclonal anti-GST (1:500) and rabbit polyclonal anti-PGP3 (1:100) were included as positive controls. Each point represents one patient and shows the mean absorbance of the two duplicate values.

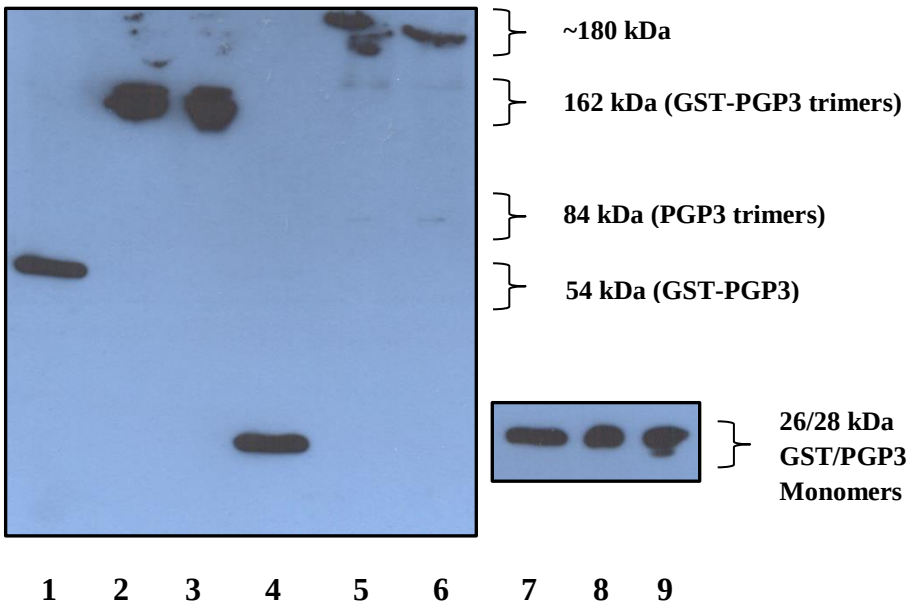


4.3.7 Effects of sodium bicarbonate buffer [pH 9.6] on the native structure of recombinant PGP3

Using Western blot, we investigated the effects of adding sodium bicarbonate buffer to the native trimer confirmation of PGP3. To mimic ELISA conditions, we diluted recombinant GST-PGP3 derived from *C. trachomatis* pSW2, cleaved PGP3 derived from *C. trachomatis* pSW2 and GST to 5 µg/ml (500 ng/well equivalent in ELISA) in 5 mM sodium bicarbonate coating buffer [pH 9.6] for 2 hours at 37°C. Recombinant proteins were also diluted in PBS for 2 hours at 37°C as a control. Aliquots were initially analysed by SDS-PAGE (2.10) but protein bands were not clearly visible due to the low concentration of protein (not shown). The experiment was repeated and results were instead analysed by western blot (2.10.8) using 12.5% SDS gels. 30 µl aliquots of GST-PGP3 SW2, PGP3 SW2 and GST at 5 µg/ml were mixed with 10 µl of either native or SDS loading buffers (2.10.2), analysed by SDS-PAGE, transferred to a PVDF membrane and incubated with polyclonal rabbit anti-PGP3 IgG at 1:100. Antibodies were then detected using an ECL kit as described (2.10.8). After two hours at 37°C, the trimeric structures of GST-PGP3 and PGP3 in both PBS and sodium bicarbonate buffer [pH 9.6] were observed (lanes 2-3, and 5-6, Figure 4.8). Only monomeric structures of GST were observed in PBS and sodium bicarbonate buffer (lanes 7-9, Figure 4.8). Although weak bands of the expected size of trimeric PGP3 SW2 (84 kDa) were observed, aggregates at approximately 180 kDa were observed after treatment with bicarbonate buffer and PBS (lanes 5 and 6, Figure 4.8). However, only the expected sizes of trimeric GST-PGP3 (162 kDa) were observed (lanes 2 and 3, Figure 4.8).

Figure 4.8. Effects of sodium bicarbonate buffer [pH 9.6] on the native structure of recombinant PGP3.

10 µl aliquots (37 ng or 5 µg/ml) of recombinant GST-PGP3 derived from *C. trachomatis* pSW2, PGP3 derived from *C. trachomatis* pSW2 and GST were analysed by SDS-PAGE on 12.5% SDS gels, transferred to a PVDF membrane, blocked with 10% skimmed milk, incubated with polyclonal rabbit anti-PGP3 (SW2) IgG at 1:100, monoclonal anti-GST IgG at 1:500 (GST antigen), anti-rabbit/mouse IgG-HRP at 1:2000 and then visualised using an ECL kit. Recombinant proteins were diluted to 5 µg/ml in 5 mM sodium bicarbonate buffer [pH 9.6] or PBS (control) for 2 hours at 37°C. Proteins were denatured (2% SDS, 1.25% BME, heat boiled for 5 minutes) to observe monomeric structures. Proteins in bicarbonate buffer or PBS were left untreated. [**Lane 1**: GST-PGP3 SW2 (SDS buffer-treated); **Lane 2**: GST-PGP3 SW2 in PBS (native buffer-treated); **Lane 3**: GST-PGP3 SW2 in bicarbonate buffer (native buffer-treated); **Lane 4**: PGP3 SW2 (SDS buffer-treated); **Lane 5**: PGP3 SW2 in PBS (native buffer-treated); **Lane 6**: PGP3 SW2 in bicarbonate buffer (native buffer-treated); **Lane 7**: GST (SDS buffer-treated); **Lane 8**: GST in PBS (native buffer-treated); **Lane 9**: GST in bicarbonate buffer (native buffer-treated)].



4.3.8 Optimisation and detection of recombinant proteins using Western blots and patient sera

During initial Western blot evaluation experiments using patient sera, many initial difficulties in reducing false-positive background (or noise) were encountered. During these studies, 1 µg of recombinant GST-PGP3 from *C. trachomatis* pSW2 was denatured in SDS loading buffer, analysed by SDS-PAGE (2.10) and transferred to a PVDF membrane, as described (2.10.7). 1% Hammarsten-grade casein, 5% BSA and 10% milk in 0.1% PBS-T were assessed in their ability to reduce false-positive background from human sera and antibodies were detected using an ECL kit, as described (2.10.8). All blocking agents resulted in similar levels of false positive background (not shown). However, very minimal false-positive background was encountered using rabbit polyclonal anti-PGP3 antisera and mouse monoclonal anti-GST/his IgG.

As background cannot be 'corrected' in a Western blot, a method was developed to reduce false positive background for each serum sample. Firstly, sera were assayed at a 1:100 dilution using an ECL kit, as described (2.10.8.3). If the resulting background was not reduced, the Western blot was repeated using a serum dilution of 1:200. Using the ECL detection method, the time that membranes were exposed to X-ray film also varied. If serum samples contained a high number of antibodies and were reactive to a recombinant protein, as little as 5 seconds was required for the exposure time. If this was still unsuccessful, the Western blot was repeated using a serum dilution of 1:100 but antibodies were instead detected using a colorimetric system (2.10.8.2). This method is less sensitive than the ECL detection system. However, as soon as the relevant protein bands appear on the membrane, the reaction can be stopped before false-positive background appears. The following experiments were conducted during these optimisation experiments and the results all contributed to the final Western blotting method using human serum samples.

4.3.8.1 Assessment of control serum reactivity

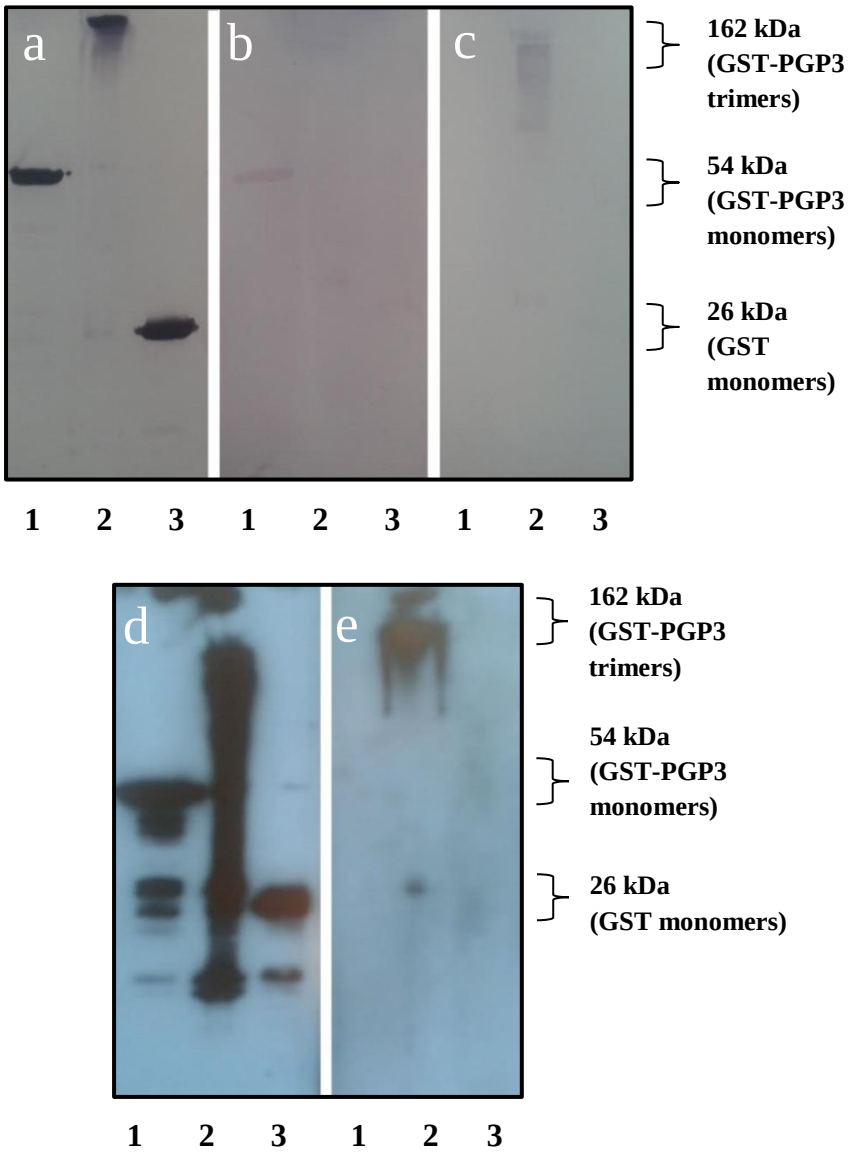
As described in 4.3.3-4.3.6, sera from non-GUM patients were used as negative controls for the optimisation of PGP3 ELISAs and Western blots as these sera were considered to be less likely to contain PGP3 antibodies than GUM sera. A total of 32 non-GUM patient sera were used in these indirect ELISA optimisation experiments. In an ELISA,

two serum samples returned a background-corrected OD_{450} value above 0.73 when assayed on two batches of GST-PGP3 derived from *C. trachomatis* pSW2 (Table 4.1). In contrast, the sera returned background-corrected OD_{450} values below 0.209 against the GST negative control antigen in an ELISA. This was investigated further using Western blot assays. 1 µg aliquots of recombinant GST-PGP3 from *C. trachomatis* pSW2 and GST were denatured in SDS loading buffer or left untreated (2.10.2), analysed by SDS-PAGE on 12.5% SDS gels (2.10), transferred to a PVDF membrane (2.10.7), and incubated with patient sera at 1:100 and anti-human IgG-HRP/AP at 1:2000. Membranes were then visualised using a colorimetric system (2.10.8.2) or an ECL kit (2.10.8.3) (Figure 4.9). Both serum samples did not react with the GST negative control. Serum from patient no. 221 resulted in a very high false positive background when using the ECL detection system (not shown). However, this serum reacted weakly to monomeric GST-PGP3 using the colorimetric system (Figure 4.9 panel (b)). Serum from patient no. 234 reacted with trimeric GST-PGP3 in both membranes visualised using the colorimetric system (Figure 4.9 panel (c)) and the ECL kit (Figure 4.9 panel (e)). The details and ELISA OD_{450} values of these sera samples can be seen in Table 4.1.

Table 4.1. Sera from non-GUM patients that returned a high OD_{450} value in the GST-PGP3 ELISA. OD_{450} values shown are the background-corrected mean absorbance of two duplicate values of sera assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST (negative control) in an indirect ELISA.

No.	Sex	OD_{450}	
		GST-PGP3 SW2	**GST (4T1/ 6P1)**
221	F	4T1 Batch 2: **0.839** 4T1 Batch 3: **0.811**	4T1 Batch 2: **0.178** 6P1 Batch 2: **0.209**
234	F	4T1 Batch 2: **0.793** 4T1 Batch 3: **0.733**	4T1 Batch 2: **0.107** 6P1 Batch 2: **0.018**

Figure 4.9. Human antibody recognition of monomeric and trimeric PGP3 in a Western blot using sera from non-GUM patients. 1 µg of recombinant GST-PGP3 derived from *C. trachomatis* pSW2 was denatured in SDS loading buffer (lane 1) or untreated (lane 2), analysed by SDS-PAGE on a 12.5% SDS gel, transferred to a PVDF membrane, incubated with patient sera at 1:100 and anti-human IgG-HRP/AP (1:2000). GST was included as a negative control (lane 3) and was heat treated with SDS. Membranes were visualised using a colorimetric system (panels (a)-(c)) or an ECL kit (panels (d)-(e)). Mouse monoclonal anti-GST (1:500) was used as a positive control (panel (a) and (d). [Lane 1: Monomer of GST-PGP3 SW2; Lane 2: trimer of GST-PGP3 SW2; Lane 3: GST (negative control)] [Panel (a): mouse monoclonal anti-GST IgG (positive control); Panel (b): no.221; Panel (c): no. 234; Panel (d): mouse monoclonal anti-GST IgG (positive control); Panel (e): no. 234].

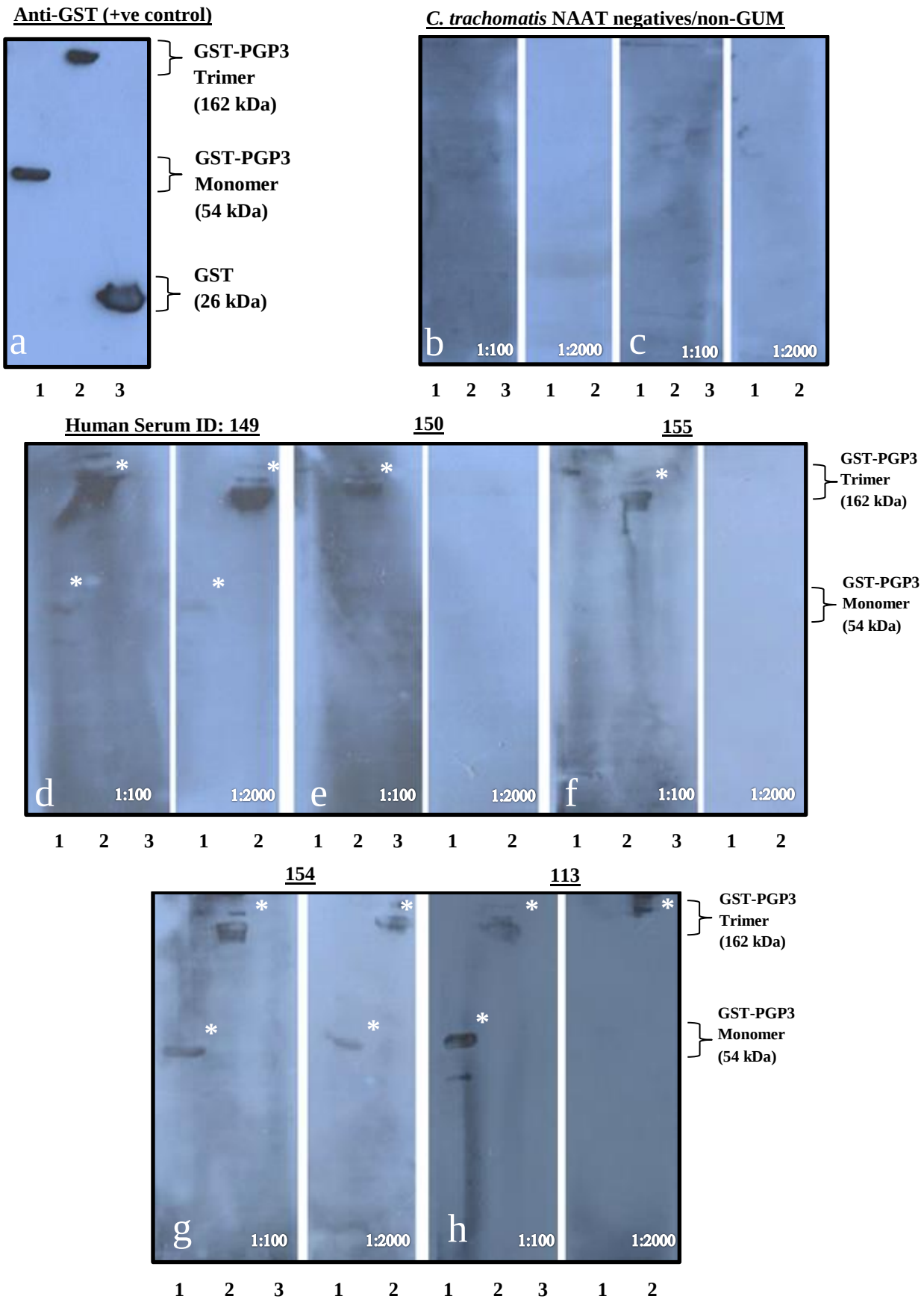


4.3.8.2 Antibody recognition of monomeric and trimeric recombinant PGP3 using sera from GUM patients

Previous reports have suggested that human antibody recognition is dependent on the native trimeric confirmation of PGP3 as these antibodies only recognise the native conformation of PGP3 (124, 125). None of the sera tested in the quoted studies reacted with the monomer of PGP3. By contrast, although we used different serum dilutions, our initial optimisation experiments using Western blots revealed that several patient sera reacted with the monomer and/or the trimer of the GST-PGP3 SW2 antigen. To analyse this discrepancy in detection, we repeated their experiments using the chemiluminescence detection system and instead used human serum dilutions of 1:100 and 1:2000. We selected human sera positive for anti-PGP3 antibodies based on their reaction with the GST-PGP3 antigen derived from *C. trachomatis* pSW2 in our ELISAs (4.3.3). We also used a pooled sample of sera from non-GUM patients and sera from GUM patients diagnosed as negative for *C. trachomatis* by NAAT as negative controls (4.2.1.1). Previous Western blot assay experiments during optimisation revealed the ability of our GST-PGP3 SW2 antigen to form a monomer and trimer under different treatments (Figure 3.15). To investigate whether serum dilution was a factor, 1 µg aliquots of recombinant GST-PGP3 from *C. trachomatis* pSW2 and GST were denatured in SDS loading buffer or left untreated (2.10.2) and analysed by SDS-PAGE on 12.5% SDS gels (2.10), transferred to a PVDF membrane (2.10.7), and blotted with human sera. Antibodies were detected using an ECL kit as described (2.10.8.3). GST was selected as a negative control antigen. Not all of the human sera assessed reacted with the monomeric or trimeric conformation of GST-PGP3 SW2. Five sera that revealed a reaction with the monomer and/or trimer of GST-PGP3 SW2 are shown in Figure 4.10, as marked by the white asterisks. At a serum dilution of 1:100, 5 sera reacted with the trimeric form of GST-PGP3 SW2, and 3 sera reacted with the monomer of GST-PGP3 SW2. None of the sera reacted with GST. At a serum dilution of 1:2000, the lowest serum dilution used by Chen *et al.* (124), 3 human sera reacted with the trimeric form of GST-PGP3 SW2, whilst 2 human sera reacted with the monomeric form of GST-PGP3 SW2. None of the non-GUM sera or sera from GUM patients diagnosed as *C. trachomatis* NAAT negative reacted with the GST-PGP3 SW2 or GST antigens.

Figure 4.10. Human antibody recognition of monomeric and trimeric PGP3 in a western blot using patient sera.

1 µg recombinant GST-PGP3 derived from *C. trachomatis* pSW2 was denatured in SDS loading buffer (lane 1) or left untreated (lane 2), analysed by SDS-PAGE on a 12.5% SDS gel, transferred to a PVDF membrane and incubated with patient sera at 1:100 and anti-human IgG-HRP (1:2000). GST was also included as a negative control (lane 3) and was heat treated with SDS. A mouse monoclonal antibody to GST (mAb anti-GST) was used as a positive control (panel a). Membranes were visualised using an ECL kit. Sera were assayed at 1:100 and 1:2000 dilutions. 10 pooled sera from GUM patients diagnosed negative for *C. trachomatis* by NAAT (panel b) and 5 pooled sera from non-GUM patients (panel c) were used as negative controls. 12 sera from GUM patients known to react with PGP3 in our ELISA were blotted against GST-PGP3 and positive reactions are marked by a white asterisk and shown in panel d-h. Note that panels d, g and h show positive reactions with the monomer of PGP3. No sera reacted with the GST negative control.


Anti-GST (+ve control)
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
GST (26 kDa)
a
1 2 3
C. trachomatis NAAT negatives/non-GUM
b 1:100 1:2000 c 1:100 1:2000
1 2 3 1 2 1 2 3 1 2
Human Serum ID: 149
150
155
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
d 1:100 1:2000 e 1:100 1:2000 f 1:100 1:2000
1 2 3 1 2 1 2 3 1 2 1 2 3 1 2
154
113
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
g 1:100 1:2000 h 1:100 1:2000
1 2 3 1 2 1 2 3 1 2

4.4 DISCUSSION

The work in this chapter describes the optimisation of ELISAs and Western blot assays for the detection of anti-PGP3 antibodies using a pilot set of patient serum samples. Recombinant his- and GST-tagged PGP3 derived from *C. trachomatis* plasmids pSW2 (serovar E) and pLGV440 (serovar L1) were also assessed for their ability to detect anti-PGP3 antibodies in ELISA and Western blot using a small cohort of selected serum samples collected from patients with a known *C. trachomatis* status, as diagnosed by NAAT.

4.4.1 Choice of blocking agent and conditions

Similar to Wills *et al.* (2009), Hammarsten-grade casein was used to block unbound sites on ELISA plates as it was revealed to be the most effective blocking buffer at reducing high background. The preparation of this blocking buffer was an initial concern, as the agent is purchased in a powered form and once in solution it must be either heated until completely dissolved or dissolved in 1M NaOH overnight (eBioscience communication). Adjusting the pH was unfavourable as it may alter the native structure of recombinant PGP3 achieved during purification. Therefore, the decision was made to heat the casein until completely dissolved and then allow it to cool to room temperature before adding to ELISA plates. However, the manufacturer's advice was to heat the casein until 70°C but only partial solubility of the casein was observed. Hammarsten casein prepared by heating to 70°C and 100°C were then compared in their ability to reduce false-positive background in an ELISA using recombinant PGP3 antigen-coated plates and human serum samples with a known reaction to PGP3 in an ELISA. No statistically significant differences were found between OD_{450} values obtained from sera assayed on plates blocked with Hammarsten casein prepared by heating to 70°C or 100°C. This experiment allayed concerns that different operators may prepare the blocking buffer to different temperatures which would potentially result in higher inter- and intra-operator variation between assays, as described further in chapter five.

4.4.2 Control serum samples

To test the ability of our recombinant PGP3 antigens to detect anti-PGP3 antibodies in patient sera, 'positive' control serum samples were selected from GUM patients who

had been diagnosed for *C. trachomatis* by NAAT and had at least one previous positive diagnosis. 'Negative' control serum samples were selected from patients with no history of a *C. trachomatis* diagnosis. However, serum from GUM patients were presumably sexually active and at risk of previous *C. trachomatis* exposure. Therefore, serum samples collected from these patients may be positive for anti-PGP3 antibodies. To increase the chance of finding 'true' negative serum controls, 32 sera from non-GUM patients were used as it was assumed that these serum samples would be less likely to contain anti-PGP3 antibodies. However, two serum samples from this cohort returned OD_{450} values above 0.733 in our GST-PGP3 SW2 ELISA which were also positive by Western blot assay.

4.4.3 Comparison and performance of different PGP3 fusion proteins in ELISAs

Control serum samples were assayed against recombinant his- and GST-tagged PGP3 derived from *C. trachomatis* pSW2 and pLGV440 in an ELISA. Recombinant his-3C MNV protease and GST were also included as negative control proteins to assess potential false-positive reactivity to the his- and GST- tags, respectively. In an ELISA, sera collected from patients who had tested positive for *C. trachomatis* by NAAT reacted with his-PGP3 derived from pSW2 but not his-PGP3 derived from pLGV440. Furthermore, the same sera reacted with GST-PGP3 derived from both *C. trachomatis* pSW2 and pLGV440. Interestingly, as described in chapter three, trimerisation of his-PGP3 derived from pSW2 was observed in Western blot, whereas his-PGP3 derived from pLGV440 did not trimerise. However, trimerisation of GST-PGP3 derived from both *C. trachomatis* pSW2 and pLGV440 was observed. These results support findings by Chen *et al.* (124)and Li *et al.* (125)that human antibody recognition of PGP3 is dependent on the native trimeric conformation of PGP3.

4.4.4 Assay saturation

In the pilot antigen ELISA studies above, sensitivities and specificities were not taken into account due to the small cohort of serum samples used. Furthermore, when new batches of recombinant PGP3 were used in an ELISA, OD_{450} values obtained from sera collected from patients diagnosed positive for *C. trachomatis* by NAAT were higher than values obtained using the first batches of recombinant proteins. It was discovered that increasing the antigen concentration in the ELISA increased the OD_{450} values obtained from positive control serum samples and antibodies but OD_{450} values obtained

from negative control serum samples did not increase. Therefore, increasing the antigen coating concentration had the potential to increase the overall sensitivity of the assay. As discussed, an antigen coating concentration described by Wills *et al.* (118) was used as an initial guide during optimisation. It was noted that the Wills *et al*. ELISA used an antigen coating concentration of 20 ng/well which was later increased to 100 ng/well for a later study (165), although the reasons for increasing the antigen coating concentration in the later study were not given. It is essential to coat the assay using the antigen at saturation, as calculated by clearly defined positive and negative controls. In the Wills *et al.* (2009) ELISA, increasing the antigen concentration would allow the detection of lower concentrations of antibodies to the antigen in sera, which would effectively create a new assay. However, the cut-off point of which a positive serum sample was not unaltered or re-calculated. This questions the reliability of the seroprevalence studies which were later undertaken. Following these findings, saturation tests were performed on each new batch of recombinant antigen.

4.4.5 Antigen storage and coating conditions

We raised concerns about the denaturing alkaline pH of the sodium bicarbonate coating buffer (pH 9.6) in the ELISA which may potentially alter the native structure of the recombinant PGP3. This may affect antibody recognition in the ELISA, as previous reports have suggested that human antibody recognition is dependent on the native trimeric conformation of PGP3 (124, 125). Recombinant PGP3 and GST-PGP3 derived from *C. trachomatis* pSW2 were diluted to 5 µg/ml in sodium bicarbonate coating buffer or PBS to mimic the 500 ng/well antigen coating concentration used in the ELISA. Western blot assay revealed no changes to the native trimeric structure of recombinant PGP3 were observed. However, these findings may not replicate the structure of recombinant PGP3 on the ELISA plate as hydrophobic interactions between soluble proteins and styrene residues on the plate may alter the final protein structure in the plate wells. Furthermore, although their reason is unclear, Wills *et al.* added 5 mM DTT in their antigen solution. As DTT disrupts disulphide bonds in protein structures, the trimeric structure of recombinant PGP3 may be affected. To investigate the effects if DTT, recombinant GST-PGP3 derived from *C. trachomatis* pLGV440 and pSW2 with or without added 5 mM DTT was assessed in their ability to detect anti-PGP3 antibodies in human serum samples. No statistically significant differences were found between OD_{450} values obtained from sera assayed against recombinant antigen with or

without added DTT. Therefore, DTT was not added to the storage buffer of the recombinant proteins.

4.4.6 Background correction and reduction

As previously discussed, there is significant variation in the methods used to detect anti-PGP3 antibodies in patient serum samples using serological assays. This includes the type of serological assay, the method of background correction, the PGP3 *C. trachomatis* serovar derivation, numbers and sources of positive and negative controls and methods of control serum characterisation. During the initial ELISAs to assess the reactivity of recombinant PGP3 proteins with antibodies in patient serum, many difficulties in high or false-positive background caused by patient serum samples were encountered. After assaying sera in uncoated (non-antigen coated) wells, it was discovered that each serum sample produced its own individual background reading. This was a concern, as the PGP3 ELISA described by Wills *et al.* (118) was used as an initial guide for the PGP3 ELISA, including background correction method, coating antigen concentration, method of antibody detection and primary and secondary antibody dilutions. The PGP3 ELISA by Wills *et al.* corrected for background by subtracting the average absorbance of two wells containing no serum, whereas we corrected 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 in a PGP3 ELISA has been described previously (123). Other studies describing PGP3 ELISAs did not state the method of background correction, if any method was used. As the Wills *et al.* PGP3 ELISA had not taken the individual background absorbance readings of each serum sample into account, the sensitivity and specificity of their ELISA may have inadvertently been lowered. In correlation with these findings, it was also discovered that each patient serum sample produced its own background in Western blot assays. However, as it is not possible to 'correct' for background, Western blot assays were optimised for each serum sample using an initial serum antibody dilution of 1:100 and enhanced chemiluminescence system as an initial first-step in the protocol. If high background produced by sera gave inconclusive results, Western blot assays were repeated using a higher antibody dilution of 1:200 and/or the less sensitive colorimetric detection method using alkaline phosphatase conjugate antibodies.

4.4.7 Human antibody recognition of PGP3 is not dependent on its trimeric structure

The potential of human antibodies to recognise monomeric and trimeric recombinant PGP3 was investigated using Western blot assays. Previous reports have suggested that human antibody recognition of PGP3 is dependent on the native trimeric conformation of PGP3 (124, 125). However, although their Western blots were not shown, Li *et al.* (125) reported antibody binding to monomeric PGP3 using a human serum dilution of 1:4000. Although we used different serum dilutions, our initial optimisation experiments using Western blot assay revealed that several patient sera reacted with the monomer and/or the trimer of the GST-PGP3 SW2 antigen. At a serum dilution of 1:100, 5 sera reacted with the trimeric form of GST-PGP3 SW2, and 3 sera reacted with the monomer of GST-PGP3 SW2. None of the sera reacted with the GST negative control antigen. At a serum dilution of 1:2000, the lowest serum dilution used by Chen *et al.* (124), 3 human sera reacted with the trimeric form of GST-PGP3 SW2, whilst 2 human sera reacted with the monomeric form of GST-PGP3 SW2. Collectively, these findings provide some reassurance regarding the native conformation of recombinant PGP3 in an ELISA, as these results suggest that human antibody recognition of PGP3 is not solely dependent on its native homotrimeric form and that binding to trimeric PGP3 may be just as likely as binding to monomeric PGP3. This is not the first time that human antibody binding to monomeric PGP3 has been reported (75, 118, 123, 125, 159, 160). Antibody binding to monomeric PGP3 in a western blot has also been reported using animal sera (160). All patient serum samples were subsequently assayed against monomeric and trimeric recombinant GST-PGP3 and the GST negative control.

4.4.2 Conclusions

- We have determined the optimal antigen format, antigen concentration and ELISA conditions for the detection of anti-PGP3 antibodies in patient serum.
- Recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 formed the most stable trimers and could detect anti-PGP3 antibodies in patient serum.
- The next step is to extend this pilot study where conditions have been optimised and compare the performance and evaluate GST-PGP3 SW2 and GST-PGP3 LGV440 using a much larger number of patient serum samples (chapter five).

This will reveal whether the assay is robust and reproducible enough to apply to a large cohort of sera i.e. a representative group, as outlined in 1.14.

CHAPTER FIVE: EVALUATION AND VALIDATION OF ELISAS USING PGP3 FROM *C. TRACHOMATIS* SEROVARS L1 AND E

5.1 INTRODUCTION

As previously discussed, the coding sequence of the 28 kDa homotrimeric PGP3 is highly conserved amongst urogenital isolates of *C. trachomatis* (126). However, CDS5 contains the most SNPs across all chlamydial serovars (Figure 3.1). In the LGV serovars of *C. trachomatis*, the PGP3 structure differs by nine amino acids from the common genital tract serovars, D-F, which result in different hydrophobic/hydrophilic characteristics. Previous studies using ELISA to detect prior *C. trachomatis* exposure have used PGP3 derived from various serovars of *C. trachomatis*, including L1 (118) and a urogenital strain, D (75, 123, 160, 163, 167, 168), which shares 100% amino acid identity to PGP3 derived from E. Furthermore, the LGV biovar of *C. trachomatis* is much less common in the general population and 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) (97). Despite this, Wills *et al.* (118) developed and evaluated a sensitive and specific ELISA to detect exposure to *C. trachomatis* using PGP3 derived from the pLGV440 (serovar L1) plasmid. The overall sensitivity and specificity of their assay was 57.9% and 97.6%, respectively. Other ELISAs incorporating recombinant PGP3 have also been described (75, 118, 123, 160, 163, 167, 168) and two have reported sensitivities and specificities as high as 95.1% (163) and 100% (168), respectively. However, variation in the number of positive and negative serum controls, the source of serum controls, methods of serum characterisation and PGP3 serovar derivation between all PGP3 ELISAs have resulted in ranges of reported sensitivities and specificities which have made the assays incomparable and unreliable.

In the previous chapter, the development and optimisation of ELISAs and Western blot assays incorporating recombinant PGP3 to detect exposure to *C. trachomatis* using patient serum samples was described. We selected recombinant PGP3 derived from *C. trachomatis* pSW2 (serovar E) and pLGV440 (serovar L1) expressed as fusion proteins with an N-terminal glutathione s-transferase (GST) tag as the coating antigen to detect anti-PGP3 antibodies. This also allows the use of GST as a negative control antigen to assess any potential false-positive reaction to the GST tag from the human

sera. 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 (185). Sera collected from patients in the United Kingdom are therefore unlikely to contain antibodies to GST. Inclusion of GST is an additional internal and rigorous control since no reaction to GST paired with any reaction to GST-PGP3 or PGP3 in an ELISA or Western blot assay 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 (124, 125, 159).

In this current chapter, the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs were used as the final preferred antigen format for validation using a larger cohort of serum samples selected from male and female GUM patients with a positive diagnosis of *C. trachomatis* by NAAT. Their PGP3 ELISA data was compared to sera from mixed-sex GUM patients with a negative *C. trachomatis* diagnosis. We also aimed to investigate the possibility that the GST tag could lower the sensitivity of the assay. We therefore repeated the ELISA using the same sets of serum samples but instead used recombinant PGP3 initially expressed as a fusion protein that was subsequently cleaved from the GST tag. Additionally, as it is impossible to accurately study the human antibody response to a natural urogenital chlamydial infection, we sought to further validate our ELISAs using sera from mice urogenitally infected with *C. trachomatis*. This would also investigate the potential of mice urogenitally infected with *C. trachomatis* to produce anti-PGP3 antibodies that would be detectable in an ELISA and Western blot assay.

To date, no serological assay incorporating PGP3 to detect anti-PGP3 antibodies in patient sera is considered to be the ‘gold standard’ serological test. However, some described ‘in-house’ assays have been used as seroprevalence tools in later studies by the same research groups (164, 165, 169). Although Goodhew *et al.* (167) reported a high sensitivity of 91.0% for their PGP3 assay, only 11 positive control serum samples were used for assay evaluation. The PGP3 ELISA described by Wills *et al.* (118) used a more rigorous evaluation method. Although they incorporated a poorly matched antigen into their assay by using recombinant PGP3 derived from the less common L1 serovar of *C. trachomatis*, they validated their assay using a high number of control serum samples in comparison to other described ELISAs (75, 123, 160, 163, 167)).

Furthermore, only Wills *et al.* demonstrated the reproducibility of their PGP3 ELISA. Assay validation is essential to be sure that the results are reliable. Therefore, we sought to demonstrate the reproducibility of our ELISAs using a large set of well-characterised serum controls and positive control antibodies.

5.1.1 Aim

- Evaluate our PGP3 ELISAs and determine the most suitable assay to detect anti-PGP3 antibodies in patient serum for seroepidemiological analyses.
    - Produce a PGP3 ELISA with an overall sensitivity and specificity higher than those reported by Wills *et al.* for their L1 PGP3 ELISA (118).

5.1.2 Objectives

- Validate our GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs using serum samples from a larger cohort of GUM patients (n=166) diagnosed as positive or negative to *C. trachomatis* by NAAT.
- Investigate the potential that the N-terminal GST tag on the GST-PGP3 fusion proteins may lower the sensitivity of the assay by repeating the ELISA using cleaved PGP3.
- Assay all serum samples against GST as a negative control to exclude the possibility of cross-reactivity to the GST tag from human serum samples.
- Calculate the specificity, sensitivity and receiver operating characteristics (ROCs) of all ELISAs.
- Assay serum samples collected from mice that had been urogenitally infected with *C. trachomatis* as a model of primary infection response.
- Demonstrate the reproducibility of our ELISAs by calculating inter- and intra-assay variation using well-characterised serum controls.

5.2 METHODS

5.2.1.1 Control antibodies and patient serum

166 unlinked and anonymised serum samples were collected from male and female GUM patients with a known *C. trachomatis* status as diagnosed by NAAT (see 4.2.1).

5.2.3 Indirect ELISAs using GUM patient sera

Medisorp flat-bottom 96-well plates were coated in 500 ng/well of recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 and cleaved PGP3 derived from *C. trachomatis* pSW2 (2.11). GST negative control antigen was also used to coat plates at 250 ng/well. GST was used to coat plates at this concentration to match the fusion tag:polypeptide ratio of the GST-PGP3. Plates were washed three times in 0.1% PBS-Tween 20 (PBS-T) and blocked in 1% Hammarsten grade sodium caseinate in PBS-T (200 µl/well) for two hours at 37°C. Plates were washed three times in PBS-T and GUM patient sera with a known *C. trachomatis* NAAT status were added in duplicate at 1:100 (50 µl/well) for 1 hour at 37°C. Plates were washed six times in PBS-T and incubated with goat anti-human IgG-HRP (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 TMB solution (eBioscience) for 10 minutes. The reaction was stopped with 50µl/well 2M H_2SO_4 and absorbance was read at OD_{450} using a BioRad iMark reader.

5.2.3.1 Background correction

The background of OD values was corrected using the method described in 4.3.2.

5.2.3.2 Detection of recombinant PGP3 in a Western blot using sera from patients

1 µg of purified recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3.1) and GST (3.3.3.2) were denatured in 1x SDS loading buffer (2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 minutes) or untreated (2.10.2). 10 µl samples were analysed on a 12.5% SDS gel (2.10) and transferred to a PVDF membrane (2.10.7). Rabbit polyclonal anti-PGP3 antisera diluted 1:1000 and mouse monoclonal anti-GST IgG diluted 1:500 were used as primary antibody controls. The dilution of human serum

used was dependent on the false-positive background produced by each sample, as explained in 4.3.7. As an initial guide, human serum samples were diluted to 1:100 in 1% skimmed milk solution and an ECL kit was used as the detection method. Antibodies were detected as described (2.10.8).

5.2.4 Indirect ELISAs using sera from mice urogenitally infected with C. trachomatis

Sera collected from ten mice urogenitally infected with *C. trachomatis* L2 P^- .pGFP::SW2 were assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3.1), PGP3 derived from *C. trachomatis* pSW2 (3.3.3.3) and GST (3.3.3.2) in an ELISA. As a negative control, we also assayed ten mice urogenitally infected with *C. trachomatis* L2 P^- 25667R. All mice were inoculated using the intravaginal (IV) route using a single dose of 10^6 inclusion-forming units. Sera were collected 35 days post-inoculation and were assayed at 1:50 in duplicate using the ELISA protocol and background-correction method as described in 5.2.3. Plates were coated using 500ng/well of recombinant GST-PGP3 SW2 and PGP3 SW2 and 250 ng/well of GST negative control antigen, as described (2.11). Serum samples were shipped on dry ice and stored at -20°C. The use of mice sera was previously approved by the Midwestern University Institutional Animal Care and Use Committee (135) and were kindly donated by Professor Kyle Ramsey.

5.2.5 Detection of recombinant PGP3 in a Western blot using sera from mice

Sera that returned a high background-corrected OD_{450} value in the PGP3 ELISAs were blotted against monomeric and trimeric recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST negative control antigen using a 1:100 serum dilution. 1 µg aliquots of recombinant protein were denatured in 1x SDS loading buffer (2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 minutes) or left untreated (2.10.2). 10 µl aliquots were analysed by SDS-PAGE on 12.5% SDS gels (2.10) and transferred to a PVDF membrane for Western blotting, as described (2.10.8). Antibodies were detected using an ECL kit.

5.2.6 Reproducibility of the GST-PGP3 SW2 ELISA

5.2.6.1 Controls

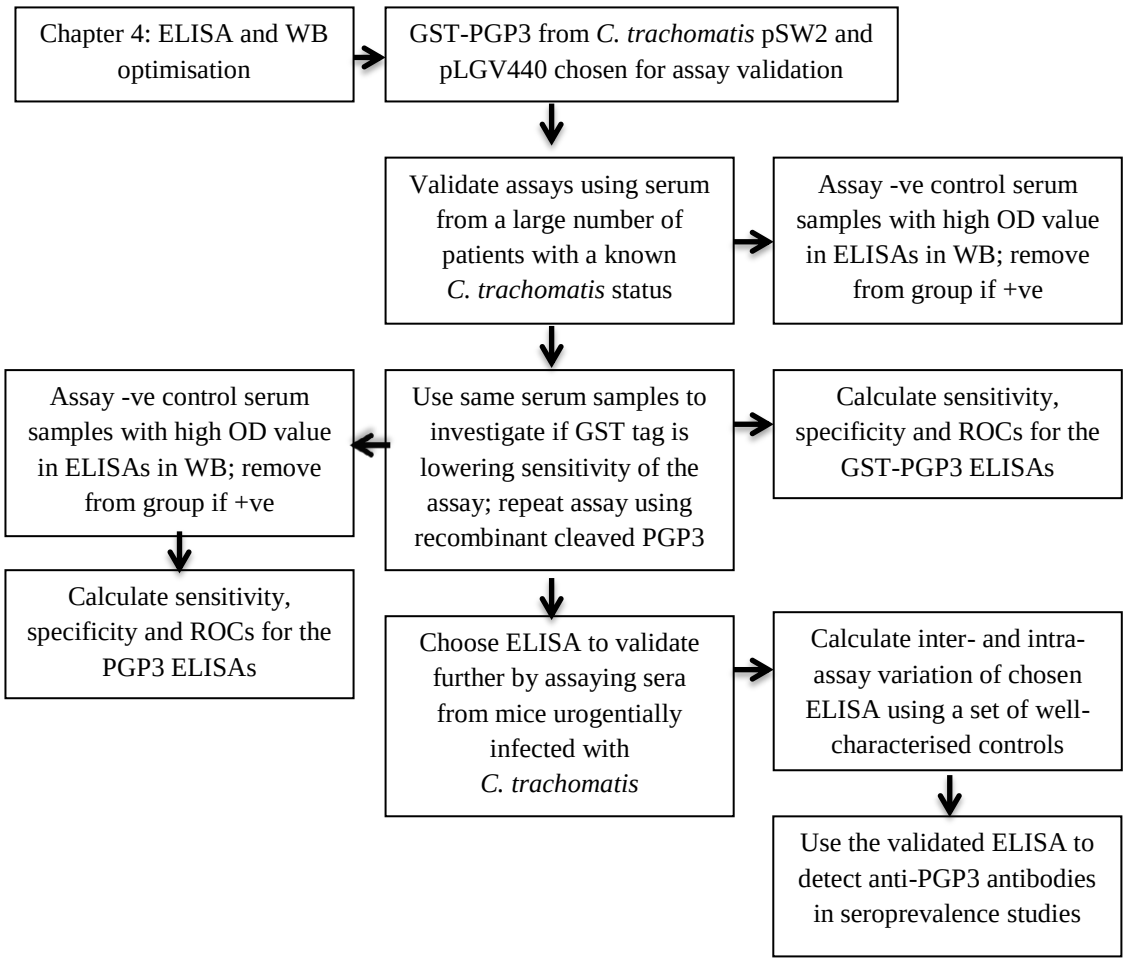
We selected three patient sera (4.2.2.1) known to react to GST-PGP3 derived from *C. trachomatis* pSW2 as positive controls, as determined in previous ELISAs. These sera

were selected based on their OD_{450} range between ~0.7-3.3. We also selected three sera known not to react with GST-PGP3 SW2 in an ELISA as negative controls. These were selected based on their mean OD_{450} range between ~0.1-0.4. Sera were also selected based on their reaction to GST-PGP3 SW2 in a Western blot and samples that were abundant in volume. Mouse monoclonal anti-GST IgG (1:10,000) (Invitrogen, Life Technologies) and rabbit polyclonal anti-PGP3 IgG (1:10,000) antibodies were used as positive controls.

5.2.6.2 Method

Each control was assayed in duplicate on each plate (intra-) on each day-to-day assay (inter-assay variation) for a total of twenty independent control results using the method described in 5.2.3. Mean OD_{450} values, standard deviations and coefficient of variation for each control was calculated (2.12).

Figure 5.1. Strategy for the evaluation of GST-PGP3 and PGP3 ELISAs using human serum samples. Flowchart also shows the order at which experiments were conducted.



5.3 RESULTS

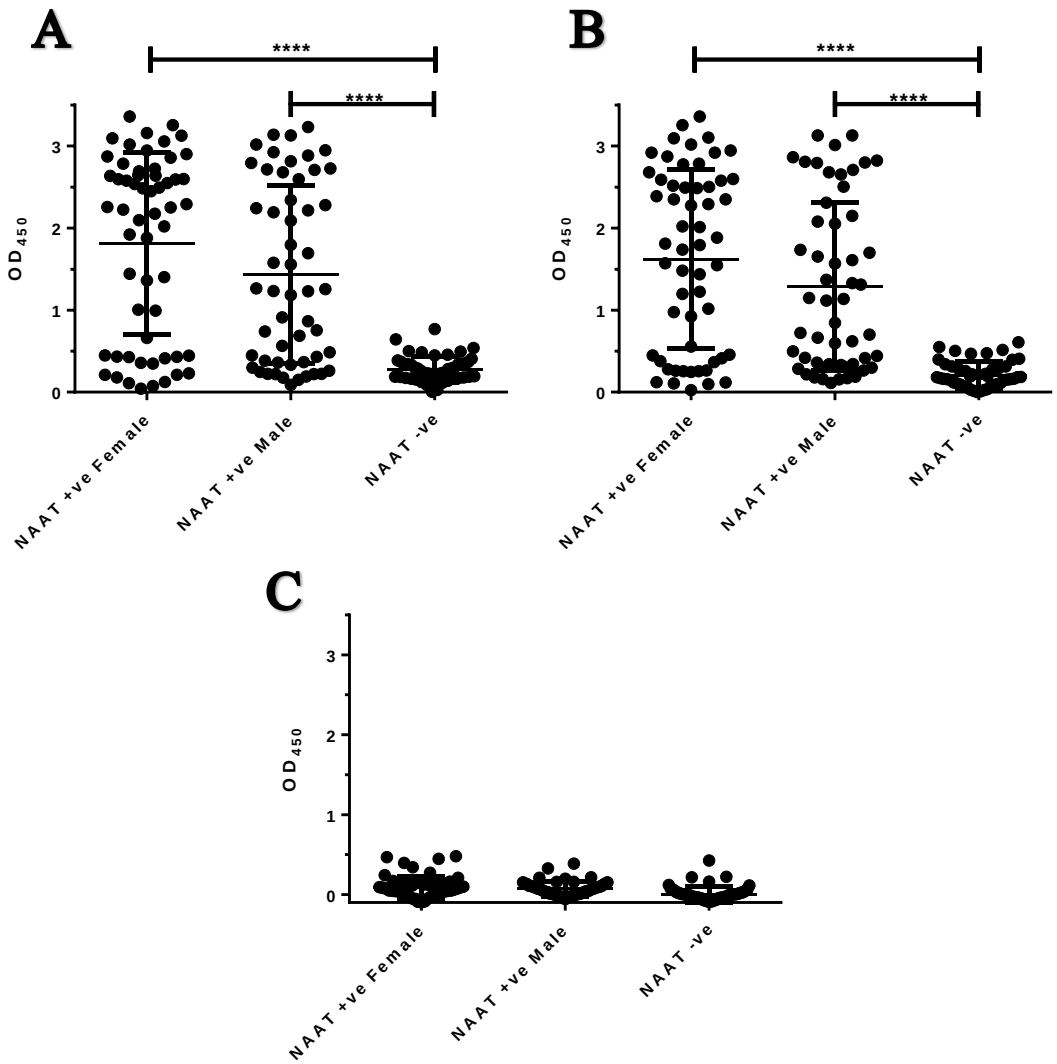
5.3.1 Detection of anti-PGP3 antibodies using patient sera and recombinant GST-PGP3 derived from C. trachomatis pSW2 and pLGV440 in an indirect ELISA

To evaluate the ELISAs, purified recombinant GST-tagged PGP3 derived from *C. trachomatis* pSW2 and pLGV440 were used at 500 ng/well using sera from 166 patients diagnosed as positive or negative for *C. trachomatis* by NAAT (5.2.1), as described 5.2.3. All sera were assayed against GST-PGP3 SW2 and GST-PGP3 LGV440 antigens at 1:100 in duplicate and OD_{450} values were corrected for background. Monoclonal mouse anti-GST IgG and polyclonal rabbit anti-PGP3 IgG antibodies were also assayed at 1:500-20,000 and 1:100-20,000, respectively, as positive controls. Mean absorbances were calculated and grouped according to the patient sex and NAAT status (Figure 5.2A and 5.2B). One-way ANOVA with Tukey multiple comparisons tests were used to compare the groups and calculate means and standard errors (2.12). Statistical significance was found between all sera from patients diagnosed positive for *C. trachomatis* by NAAT and sera from patients diagnosed negative in both GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs ($p = <0.0001$). No statistical significance was found between the sera from female and male GUM patients diagnosed positive for *C. trachomatis* by NAAT in the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs ($p = >0.05$). OD_{450} values of GST-PGP3 SW2 ELISA ranged from 0.042-3.35 (female positives; n = 57), 0.089-3.231 (male positives; n = 53) and -0.02-0.769 (mixed sex negatives; n = 50). OD_{450} values of the GST-PGP3 LGV440 ELISA ranged from -0.018-3.35 (female positives; n = 57), 0.111-3.12 (male positives; n = 53) and -0.06-0.608 (mixed sex negatives, n = 50). Six serum samples from patients diagnosed negative for *C. trachomatis* by NAAT returned a high background-corrected OD_{450} value. These OD_{450} values ranged between 0.98-2.73 for the GST-PGP3 LGV440 ELISA and 1.85-2.904 for the GST-PGP3 SW2 ELISA. However, all sera were assayed against GST as a negative control and OD_{450} values ranged from -0.029-0.127. These sera were considered to be positive for anti-PGP3 antibodies and were removed from the mixed sex negative group and statistical analyses. These sera were investigated further in western blots, described below in 5.3.3.

5.3.1.1 Glutathione s-transferase negative control

To match the tag:polypeptide ratio of the GST-PGP3 antigens used in the ELISA described above (5.3.2), glutathione s-transferase (GST) antigen was used at half the concentration of GST-PGP3 (250 ng/well) to perform an ELISA using the same GUM patient sera, as described 5.2.3. GST was selected as the negative control antigen to observe any false positive reaction from the human sera with the GST tag. Saturation tests described in 4.3.4 revealed that assay saturation was achieved at this antigen concentration. All sera were assayed at 1:100 in duplicate and OD_{450} values were corrected for background. Monoclonal mouse anti-GST IgG and polyclonal rabbit anti-PGP3 IgG antibodies were also assayed at 1:500-20,000 and 1:100-20,000, respectively, as positive controls. Mean absorbances were calculated and grouped according to the patient sex and NAAT status (Figure 5.2C). One-way ANOVA with Tukey multiple comparisons tests were used to compare the groups and calculate means and standard errors (2.12). No statistical significance was found between any sera from patients diagnosed positive for *C. trachomatis* by NAAT and mixed sex negative groups (all p = >0.05). Furthermore, no statistical significance was found between sera from patients diagnosed negative for *C. trachomatis* by NAAT in the GST, GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs (all p = >0.05). However, statistical significance was found between the sera from patients positive for *C. trachomatis* by NAAT in the GST ELISA and the same sera in the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs (all p = <0.0001). In the GST ELISA, OD_{450} values in the female positives (n = 57) ranged between -0.185-0.481. OD_{450} values in the male positives (n = 53) ranged between -0.031-0.387. Finally, OD_{450} values in the mixed sex negatives (n = 50) ranged between -0.189-0.428. All 166 GUM patient sera assayed returned an OD_{450} against GST below 0.481.

Figure 5.2. Antibody responses from GUM patient sera to recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and pLGV440 by indirect ELISA. Graphs show background-corrected mean OD_{450} values of sera collected from GUM patients with a known *C. trachomatis* status as diagnosed by NAAT. Sera were assayed in duplicate at 1:100 against GST-PGP3 derived from *C. trachomatis* pSW2 or pLGV440 (500 ng/well). All sera were also assayed against GST as a negative control (250 ng/well). Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean and standard deviation bars are shown. **** = statistical significance using Tukey multiple comparison tests from one-way ANOVA (p = <0.0001). **[A]** GST-PGP3 SW2; **[B]** GST-PGP3 LGV440; **[C]** GST (negative control). [NAAT +ve females = 57; NAAT +ve males = 53; NAAT –ve mixed = 50].



5.3.1.2 Detection of anti-PGP3 antibodies in patient sera using recombinant GST-PGP3 derived from C. trachomatis pSW2 in a Western blot

Western blots were conducted to investigate the six patient sera that returned high OD_{450} values when assayed in the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs (5.3.1), as seen in Table 5.1. 1 µg aliquots of purified recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3.1) and GST (3.3.3.2) were analysed by SDS-PAGE and blotted with patient sera at 1:100/200 (as discussed in 4.2.5). Membranes were then visualised using a colorimetric or chemiluminescence system. Some sera returned a high background on both the ECL and colorimetric western blotting systems. A mouse monoclonal anti-GST IgG antibody was used as a positive control. Sera from patient nos. 9, 28, 46 and 58 (patients diagnosed negative for *C. trachomatis* by NAAT) reacted with the monomeric GST-PGP3 SW2 protein using the colorimetric system (Figure 5.3A). However, patient nos. 11 and 61 did not react (not shown). No patient sera reacted with the GST negative control antigen (Figure 5.3B). One non-specific band is present at around 40 kDa in lanes 2 and 5, Figure 5.3A. The mouse monoclonal anti-GST IgG positive control antibody also reacted with this band, as seen in lane 1, Figure 5.3A. This band also co-elutes with recombinant GST-PGP3 SW2 and LGV440 during purification using reduced glutathione (Figure 3.12). This band is absent during the purification of GST negative control antigen using the same purification protocol (Figure 3.10).

After further optimisation and the discovery that our recombinant GST-PGP3 proteins could trimerise, these western blots were later repeated using monomeric and trimeric recombinant GST-PGP3 SW2 and the ECL system using patient sera at 1:200 (Figure 5.3C). None of the sera reacted with GST (lane 3, panels (b)-(f), Figure 5.3C). Serum no. 58 returned a very high background and the results were inconclusive (not shown). Serum nos. 9, 11, 28, 46 and 61 all displayed some background but reactions with trimeric of GST-PGP3 SW2 were still observed (lane 2, panels (b)-(f), Figure 5.3C). A mouse monoclonal anti-GST IgG was used as a positive control (panel (a), Figure 5.3C). Collectively, these patients were considered to be positive for anti-PGP3 antibodies and were removed from further ELISAs and statistical analyses in the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs (5.3.1).

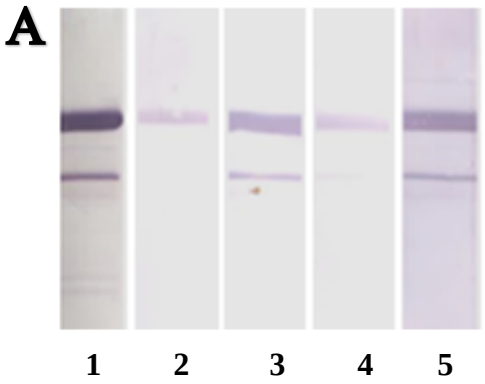
Table 5.1. Details of sera from patients diagnosed *C. trachomatis* negative by NAAT with high OD_{450} values in GST-PGP3 ELISAs. Sera were assayed at 1:100 against 500 ng/well GST-PGP3 SW2 and GST-PGP3 LGV440 and 250 ng/well GST. OD_{450} values shown are mean absorbance of two duplicate background-corrected values.

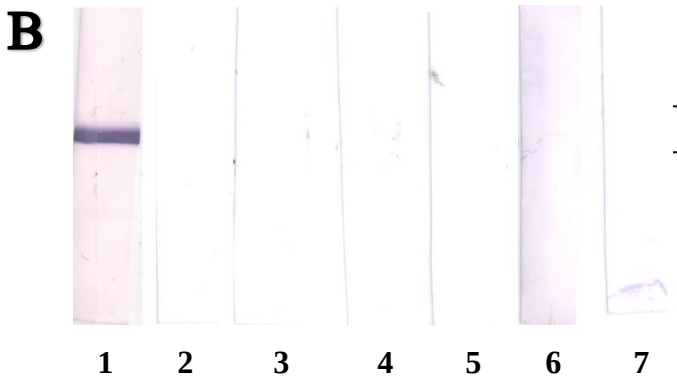
[a] = Previous history of *C. trachomatis* diagnosis by NAAT if tested at Southampton General Hospital

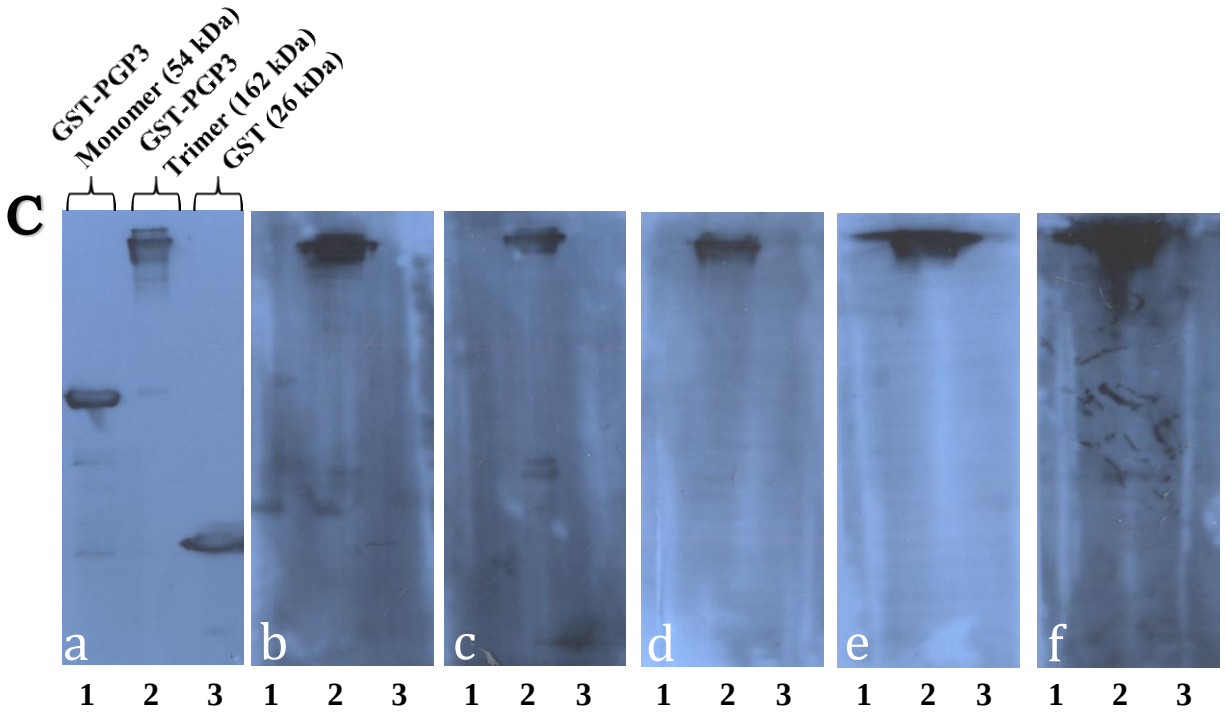
Patient No.	Previous history[a] and result (-/+)	GST-PGP3 SW2 ELISA	GST-PGP3 LGV440 ELISA	GST ELISA (-ve control)
9	N/A	2.31	2.20	-0.029
11	N/A	1.85	1.46	-0.004
28	2005 (-); 2010 (+)	1.99	1.70	-0.066
46	N/A	2.75	2.61	0.066
58	N/A	2.26	0.529	0.011
61	N/A	2.90	2.73	0.127

Figure 5.3. Human antibody recognition of recombinant GST-PGP3 in a Western blot using sera from patients diagnosed negative for *C. trachomatis* by NAAT.

1 µg aliquots of recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST were analysed by SDS-PAGE, blotted with patient sera at 1:100 **[A/B]** 1:200 **[C]** and visualised using a colorimetric or ECL kit. **[A]** GST-PGP3 SW2 was denatured (2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 minutes) and bound antibodies were detected using the colorimetric system [Lane 1: mouse monoclonal anti-GST IgG (positive control); Lane 2: patient no. 9; Lane 3: patient no. 28; Lane 4: patient no. 46; Lane 5: patient no. 58] **[B]** GST was denatured as described and bound antibodies detected using the colorimetric system. [Lane 1: mouse monoclonal anti-GST IgG (positive control); Lane 2: patient no. 9; Lane 3: patient no. 11; Lane 4: patient no. 28; Lane 5: patient no. 46; Lane 6: patient no. 58; Lane 7: patient no. 61] **[C]** GST-PGP3 SW2 was denatured as described (lane 1) or left untreated (lane 2). GST was included as a negative control (lane 3) and denatured by treating as described. Membranes were visualised using the ECL system [Panel (a): mouse monoclonal anti-GST IgG (positive control); (b): patient no. 9; (c): no. 11; (d): no. 28; (e): no. 46; (f) no. 61.


A
54 kDa
(GST-PGP3
monomer)
1
2
3
4
5


B
26 kDa
(GST
monomer)
1
2
3
4
5
6
7


C
GST-PGP3
Monomer (54 kDa)
GST-PGP3
Trimer (162 kDa)
GST (26 kDa)
a
b
c
d
e
f
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

5.3.1.3 Sensitivity and specificity of the GST-PGP3 ELISAs and determination of cut-off points

GraphPad Prism version 6.0 was used to calculate sensitivities and specificities of the GST-PGP3 ELISAs using Receiver Operating Characteristic (ROC) curve analyses (2.12). Results were considered to be statistically significant if p = <0.05. The high OD_{450} values obtained for the 6 sera from patients diagnosed positive for *C. trachomatis* by NAAT (described in 5.3.1.2) were considered positive for anti-PGP3 antibodies and were not included in the statistical analyses. The ROC and statistical analyses can be seen in Table 5.2. For the GST-PGP3 SW2 ELISA, cut-off points of OD_{450} values 0.652 and 0.665 were selected for females and males, respectively, to give a specificity of 98.0% for both groups. Sensitivities of 71.93% and 64.15% were obtained for female and male NAAT positive groups, respectively. For the GST-PGP3 LGV440 ELISA, cut-off points of OD_{450} values 0.553 and 0.573 were selected for females and males, respectively, to give a specificity of 98.0% for both groups. Lower sensitivities of 70.18% and 62.26% were obtained for female and male NAAT positive groups, respectively.

Table 5.2. Sensitivities and specificities of the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs. 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.

GST-PGP3 SW2	**Cut off OD_{450}**	**ROC Area**	**P value**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
Female	0.652	0.864	<0.0001	71.93 (58.46-83.03)	98.00 (89.35-99.95)
Male	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)
GST-PGP3 LGV440	**Cut off OD_{450}**	**ROC Area**	**P value**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
Female	0.553	0.870	<0.0001	70.18 (56.60-81.57)	98.00 (89.35-99.95)
Male	0.573	0.879	<0.0001	62.26 (47.89-75.21)	98.00 (89.35-99.95)
Both	0.553	0.874	<0.0001	66.36 (56.73-77.02)	98.00 (89.35-99.95)

5.3.2 Detection of anti-PGP3 antibodies using patient sera and PGP3 derived from C. trachomatis pSW2 in an indirect ELISA

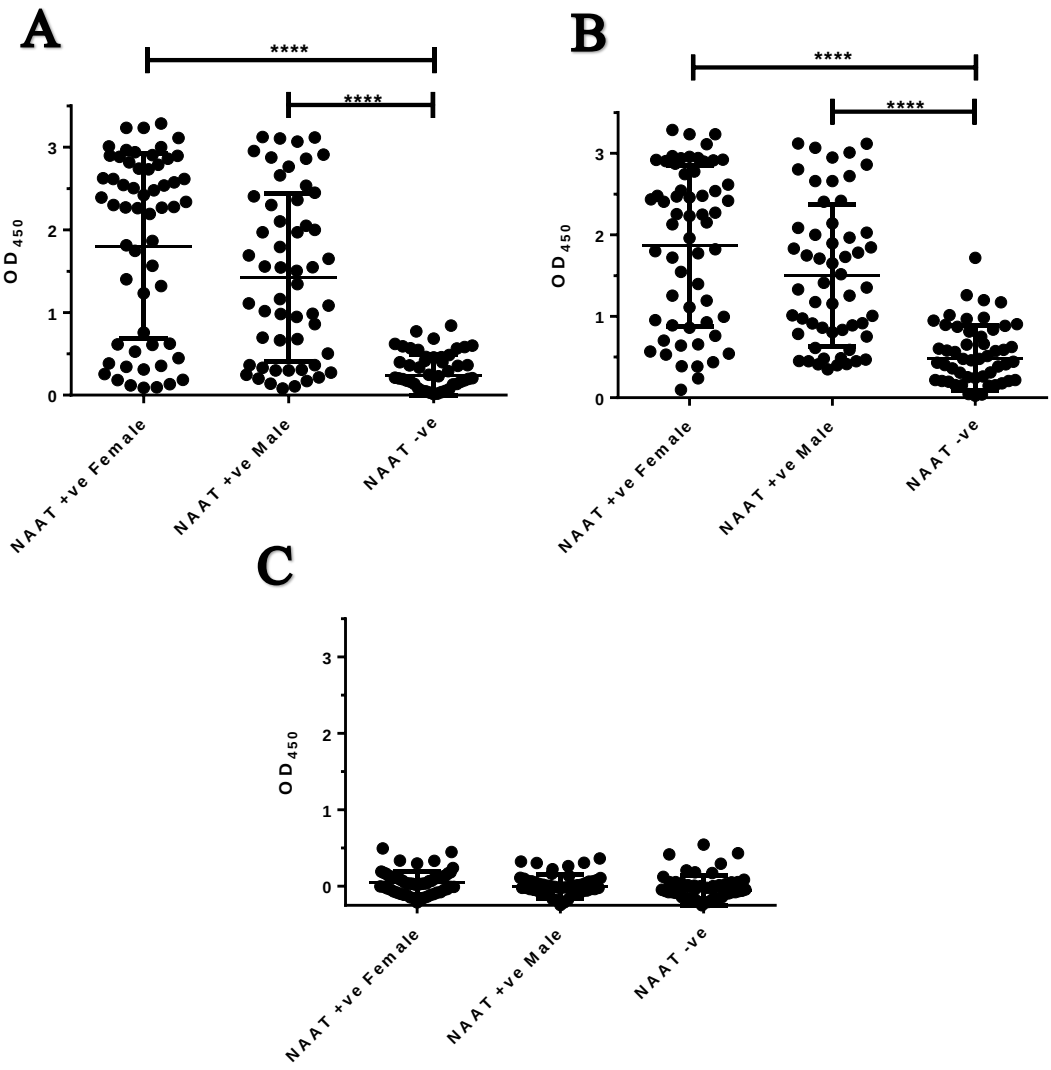
To investigate the possibility that the N-terminal GST tag on recombinant GST-PGP3 was affecting the sensitivity of the ELISA, the above experiment (5.3.1) was repeated using PGP3 derived from *C. trachomatis* pSW2 that had subsequently been cleaved from the GST tag (3.3.3.3). Purified recombinant cleaved PGP3 derived from *C. trachomatis* pSW2 was used to coat plates at 500 ng/well to perform an ELISA using sera from GUM patients diagnosed as positive or negative for *C. trachomatis* by NAAT. As the GST-PGP3 SW2 antigen was considered to be more appropriate and sensitive than the GST-PGP3 LGV440 antigen in an ELISA (as described in 5.3.1.3) for the detection of anti-PGP3 antibodies, the cleaved PGP3 SW2 was chosen to assay the same patient sera. An additional thirteen serum samples were collected after experiments described in 5.3.1 were conducted (seven samples from patients diagnosed positive for *C. trachomatis* by NAAT and six diagnosed negative). Two serum samples used in the GST-PGP3 ELISAs (5.3.1) were excluded as serum volumes were insufficient. All sera were also re-assayed against a different batch of the GST-PGP3 SW2 antigen and were also re-assayed against the GST negative control antigen. GST-PGP3 SW2, PGP3 SW2 and GST antigens were assayed in duplicate and OD_{450} values were corrected for background. Mean absorbances were calculated and grouped according to the patient sex and NAAT status (Figure 5.4). One-way ANOVA with Tukey multiple comparisons tests (2.12) were used to compare the groups and calculate means and standard errors. Statistical significance was found between sera from patients diagnosed positive and patients diagnosed negative for *C. trachomatis* by NAAT in both GST-PGP3 SW2 and cleaved PGP3 SW2 ELISAs ($p = <0.0001$). No statistical significance was found between female and male serum samples in the GST-PGP3 SW2 and cleaved PGP3 SW2 ELISAs ($p = >0.05$). OD_{450} values of GST-PGP3 SW2 ELISA ranged from -0.147-3.285 (female positives; n = 60), -0.057-3.121 (male positives; n = 55) and -0.17-0.835 (mixed sex negatives; n = 56). OD_{450} values of the cleaved PGP3 SW2 ELISA ranged from -0.016-3.285 (female positives; n = 60), 0.349-3.119 (male positives; n = 54) and -0.193-1.71 (mixed sex negatives, n = 56). Fifteen sera in the mixed sex NAAT negative group returned an OD_{450} value above 0.75 in the cleaved PGP3 SW2 ELISA. These OD_{450} values ranged between 0.786-1.71 for the cleaved PGP3 SW2 ELISA. However, the highest OD_{450} value obtained from these sera for the

repeated GST-PGP3 SW2 ELISA was 0.835 (0.769 in 5.3.1). These serum samples did not include the serum samples collected from patients diagnosed negative for *C. trachomatis* by NAAT that returned high OD_{450} values in the GST-PGP3 SW2 and LGV440 ELISAs, as described in 5.3.1.2. These sera were investigated further in western blots, described below in 5.3.2.2.

5.3.2.1 Glutathione s-transferase negative control

All serum samples were re-assayed against GST (3.3.3.2) as a negative control, as described (5.2.3). GST antigen was used to coat plates at 250 ng/well to perform an ELISA using the same patient sera, as described (5.2.3). All sera were assayed at 1:100 in duplicate and OD_{450} values were corrected for background. One-way ANOVA with Tukey multiple comparisons tests were used to compare the groups and calculate means and standard errors (2.12). Similar to the GST ELISA described above (5.3.1.1), no statistical significance was found between any sera from female or male GUM patients diagnosed positive for *C. trachomatis* by NAAT and mixed sex patients diagnosed negative by NAAT (all $p = >0.05$). All background-corrected OD_{450} values ranged from -0.428-0.546.

Figure 5.4. Antibody responses from GUM patient sera to recombinant GST-PGP3 and cleaved PGP3 derived from *C. trachomatis* pSW2 by indirect ELISA. Graphs show background-corrected mean OD_{450} values of sera collected from GUM patients with a known *C. trachomatis* status as diagnosed by NAAT. Sera were assayed in duplicate at 1:100 against GST-PGP3 derived from *C. trachomatis* pSW2 or PGP3 that was subsequently cleaved from the GST tag (500 ng/well). All sera were also assayed against GST as a negative control (250 ng/well). Each point represents one patient and shows the mean absorbance of the two duplicate values. Mean and standard deviation bars are shown. **** = statistical significance using Tukey multiple comparison tests (p = <0.0001). **[A]** GST-PGP3 SW2; **[B]** Cleaved PGP3 SW2; **[C]** GST (negative control). [NAAT +ve females = 60; NAAT +ve males = 55; NAAT –ve mixed = 56].



5.3.2.2 No detection of anti-PGP3 using GST-PGP3 and patient sera in a Western blot

Western blots were conducted to investigate the fifteen patient sera that returned OD_{450} values above 0.75 when assayed against recombinant cleaved PGP3 derived from *C. trachomatis* pSW2 in an ELISA (3.5.1). 1 µg aliquots of monomeric and trimeric cleaved PGP3 SW2 and GST were analysed by SDS-PAGE, transferred to a PVDF membrane, incubated with patient sera at 1:100 and anti-human IgG-HRP at 1:2000, as described (5.2.3.2). Membranes were then visualised using an ECL kit. Rabbit polyclonal anti-PGP3 positive control antibody was used at a 1:1000 dilution as a positive control. Patient sera no. 5, 6, 9, 27, 30, 34, 35, 44, 45, 49, 50, 52, 133, 137 and 138 were assayed but no reaction to either monomeric or trimeric cleaved PGP3 SW2 was observed (not shown). Furthermore, no reaction was observed to the GST negative control antigen from any of the sera (not shown).

5.3.2.3 Sensitivity and specificity of the GST-PGP3 ELISAs and determination of cut-off points

GraphPad Prism version 6.0 was used to calculate sensitivities and specificities of assays using Receiver Operating Characteristic (ROC) curve analyses (2.12). Results were considered to be statistically significant if $p = <0.05$. The ROC analyses can be seen in Table 5.3. The high OD_{450} values obtained for the 6 serum from male and female GUM patients diagnosed negative for *C. trachomatis* by NAAT (described in 5.2.1 and 5.2.3) were considered positive for anti-PGP3 antibodies and were not re-assayed or analysed in this experiment. For the repeated GST-PGP3 SW2 ELISA (5.3.2), similar sensitivity and specificity results to those in Table 5.1 were achieved Table 5.3A).. Cut-off points of OD_{450} values 0.719 and 0.734 were selected for females and males, respectively, to give a specificity of 96.43% for both female and male serum groups. Sensitivities of 71.67% and 65.45% were obtained for female and male *C. trachomatis* positive groups, respectively. These results are inclusive of the OD_{450} results of the additional sera obtained after conducting the GST-PGP3 SW2/LGV440 ELISAs for the first time. As the western blot results for the fifteen sera assayed against PGP3 SW2 were negative for any reaction to GST-PGP3 (described in 5.3.2), the OD_{450} results were included in the ROC analyses. For the PGP3 SW2 ELISA, two sets of analyses were conducted to compare results with the sensitivities and specificities of the

GST-PGP3 ELISAs (Table 5.3B). Cut-off points of OD_{450} values 1.327 and 1.293 were selected for females and males, respectively, to give a specificity of 98.21% for both groups with sensitivities of 65.0% and 52.73% obtained for female and male NAAT positive groups, respectively. Sensitivities of 71.67% and 65.45% (similar to the GST-PGP3 ELISAs) for female and male NAAT positive groups gave lower specificities of 87.50% and 85.71%, respectively.

Table 5.3. Sensitivities and specificities of the GST-PGP3 SW2 and cleaved PGP3 SW2 ELISAs.

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. For the PGP3 SW2 ELISA, two sets of analyses were conducted to compare the assay sensitivities and specificities to those of the GST-PGP3 SW2 ELISA. **[A]** GST-PGP3 SW2 ELISA; **[B]** PGP3 SW2 ELISA

A

GST-PGP3 SW2	Cut off OD_{450}	ROC Area	P value	Sensitivity (%) (95% CI)	Specificity (%) (95% CI)
Female	0.719	0.868	<0.0001	71.67 (58.56-82.55)	96.43 (87.69-99.56)
Male	0.734	0.864	<0.0001	65.45 (51.42-77.76)	96.43 (87.69-99.56)
Both	0.689	0.866	<0.0001	68.70 (59.38-77.02)	96.43 (87.69-99.56)

B

PGP3 SW2	**Cut off OD_{450}**	**ROC Area**	**P value**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
Female	1.327	0.878	<0.0001	65.00 (51.6-76.87)	98.21 (90.45-99.5)
Male	1.293	0.857	<0.0001	52.73 (38.8-66.35)	98.21 (90.45-99.5)
Both	1.293	0.868	<0.0001	59.13 (49.57-68.21)	98.21 (90.45-99.5)
PGP3 SW2	**Cut off OD_{450}**	**ROC Area**	**P value**	**Sensitivity (%) (95% CI)**	**Specificity (%) (95% CI)**
Female	1.327	0.878	<0.0001	71.67 (58.56-82.55)	87.50 (75.93-94.82)
Male	1.293	0.857	<0.0001	65.45 (51.42-77.76)	85.71 (75.93-94.82)
Both	1.293	0.868	<0.0001	67.83 (58.47-76.23)	87.50 (75.93-94.82)

5.3.3 Reproducibility of the GST-PGP3 SW2 ELISA

5.3.3.1 Inter- and intra-assay variation

Experiments were conducted to demonstrate the reproducibility of our GST-PGP3 ELISA using human serum samples and positive control antibodies (5.2.6). Six serum samples from GUM and non-GUM patients (5.2.6.1) were selected based on their known response to recombinant GST-PGP3 derived from *C. trachomatis* pSW2. These known responses were determined from many 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. Three sera (no. 215, 216, 217) were selected as negative controls and three sera (no. 204, 205, 234) were selected as positive controls and were assayed at 1:100. Mouse anti-GST monoclonal and rabbit anti-PGP3 polyclonal antibodies were selected as positive controls and each assayed at 1:10,000. Controls were assayed in duplicate on each plate (intra-) on each independent assay (inter-assay variation) for a total of twenty independent assay run results (Figure 5.5A and 5.5B). Assay numbers 1-17 were carried out by operator 1 and assays 18, 19 and 20 were carried out by operators 2, 3 and 4, respectively. Mean OD_{450} values, standard deviations, coefficient of variation and the minimum and maximum OD_{450} values obtained for each control were calculated using GraphPad Prism 6.0 (2.12) and were all calculated using the raw and background-corrected OD_{450} values obtained from the twenty independent assay runs (Table 5.4A/B and 5.5A/B, respectively). The standard deviations of all positive and negative controls across twenty independent assays ranged from 0.098-0.198 (raw OD_{450} data) and 0.032-0.195 (background-corrected OD_{450} data). Coefficient of variation (CV) was calculated using OD_{450} values of positive control sera and antibodies obtained across twenty independent assays and excluded values obtained using negative control serum samples. CV ranged between 5.61-12.39% (raw data) and 5.85-12.6% (background-corrected data) for inter-assay variation. CV ranged between 2.28-7.53% (raw data) and 2.64-7.14% (background-corrected data) for intra-assay variation for positive control sera and antibodies.

Figure 5.5. Levey-Jennings chart of twenty independent GST-PGP3 SW2 assays.

Graph shows the mean duplicate OD_{450} values of six control sera from patients (assayed at 1:100) and anti-GST and anti-PGP3 antibodies (1:10,000) assayed against GST-PGP3 SW2 antigen in duplicate across two plates over twenty independent ELISAs (Assay no.). Each point represents the mean absorbance of the two duplicate values from two plates. Assays 1-17 were carried out my operator 1, and no 18, 19 and 20 by operators 2, 3 and 4, respectively. **[A]** Raw OD_{450} data; **[B]** Background-corrected OD_{450} data.

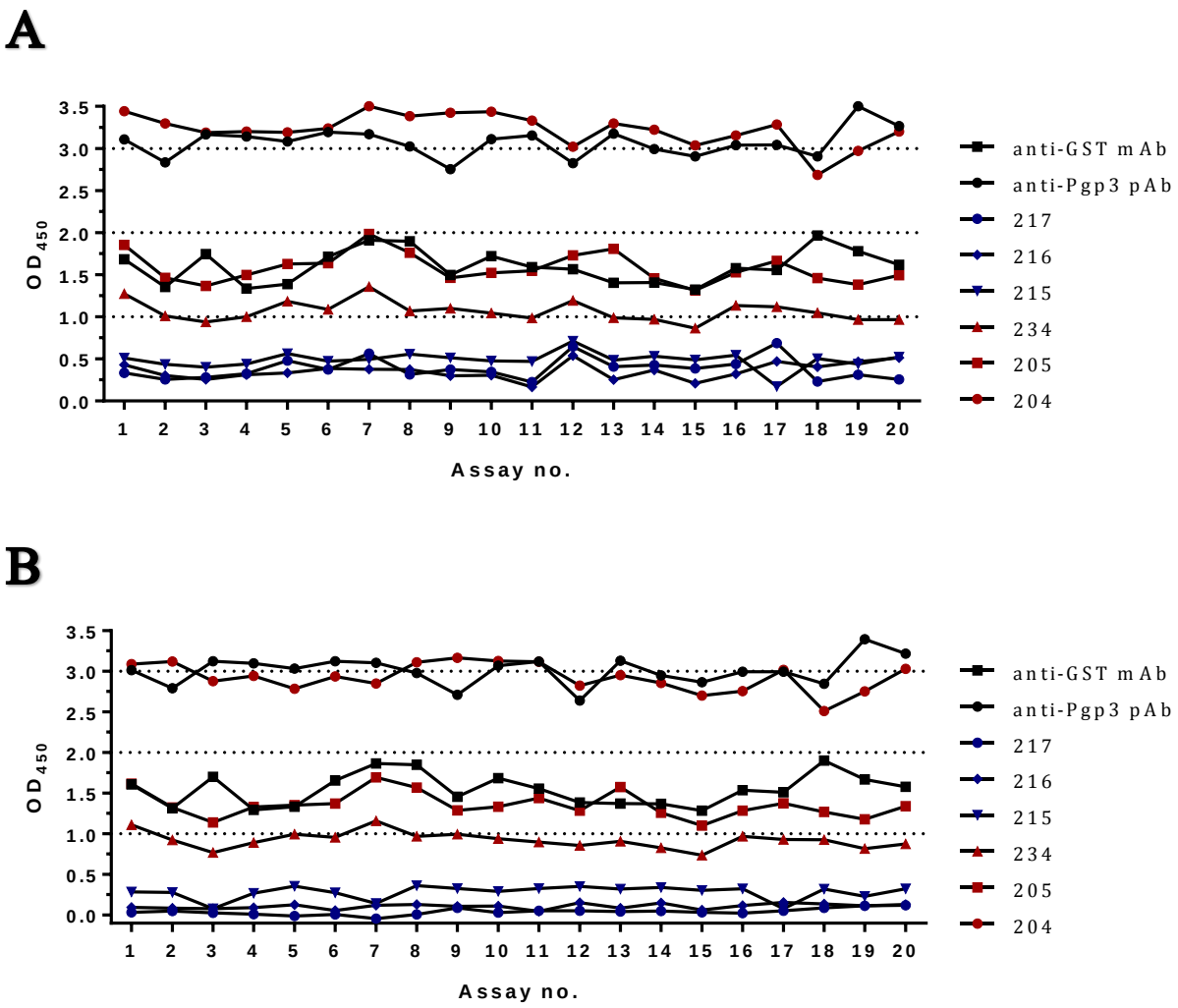


Table 5.4. Inter-assay variation of the GST-PGP3 SW2 ELISA.

Six sera from patients (assayed in duplicate at 1:100) and anti-GST and anti-PGP3 antibodies (1:10,000) were assayed against GST-PGP3 SW2 antigen in indirect ELISAs. Data were calculated using GraphPad Prism 6.0 based on means calculated from duplicate OD_{450} values from two plates over twenty independent ELISAs (n=20). Positive control sera and antibodies are in **bold**. **[A]** Raw OD_{450} data; **[B]** Background-corrected OD_{450} data

A

Serum/ Control	**204**	**205**	**234**	**215**	**216**	**217**	**Anti-PGP3**	**Anti-GST**
Mean OD_{450}	3.225	1.578	1.065	0.487	0.355	0.383	3.069	1.602
Min OD_{450}	2.685	1.314	0.865	0.174	0.167	0.225	2.754	1.322
Max OD_{450}	3.5	1.986	1.358	0.711	0.538	0.687	3.5	1.965
SD	0.192	0.177	0.120	0.098	0.098	0.129	0.172	0.198
SEM	0.043	0.039	0.027	0.022	0.022	0.029	0.039	0.044
CV (%)	**5.94**	**11.20**	**11.26**	20.13	27.60	33.62	**5.61**	**12.39**

B

Serum/ 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**

Table 5.5. Intra-assay variation of the GST-PGP3 SW2 ELISA.

Six sera from patients (assayed in duplicate at 1:100) and anti-GST and anti-PGP3 antibodies (1:10,000) were assayed against GST-PGP3 SW2 antigen in indirect ELISAs. Data were calculated using GraphPad Prism 6.0 based on means calculated from duplicate OD_{450} values from two plates over twenty independent ELISAs (n=20). Positive control sera and antibodies are in **bold**. **[A]** Raw OD_{450} data; **[B]** Background-corrected OD_{450} data

A

Serum No. /Control	Mean OD_{450}	Mean SD	Mean CV (%)
204	3.23	0.072	**2.28**
205	1.58	0.1	**6.26**
234	1.06	0.064	**6.04**
215	0.487	0.048	9.82
216	0.355	0.044	11.20
217	0.383	0.041	10.43
Anti-PGP3	3.01	0.172	**5.66**
Anti-GST	1.60	0.123	**7.53**

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**

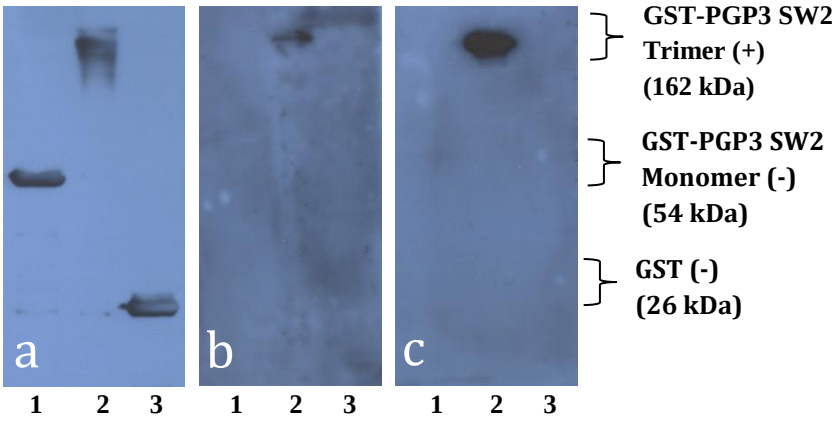
5.3.4 Detection of anti-PGP3 antibodies in an indirect ELISA using recombinant PGP3 and sera from mice urogenitally infected with C. trachomatis

Ramsey *et al.* (135) infected outbred female Swiss Webster mice with either a naturally occurring plasmid-free isolate of *C. trachomatis* from the L2 serovar (L2 P^- 25667R) or the same isolate transformed with a recombinant chlamydial plasmid, pGFP::SW2, from serovar E. This plasmid possesses CDS5 which is required for the expression of PGP3. We assayed sera collected from ten mice inoculated with *C. trachomatis* L2 P^-.pGFP::SW2 against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (500 ng/well), PGP3 derived from *C. trachomatis* pSW2 (500 ng/well) and GST (250 ng/well) in an ELISA (5.2.4). As a negative control, we also assayed ten mice inoculated with *C. trachomatis* L2 P^- 25667R. These mice were inoculated using the intravaginal route using a single dose of 10^6 inclusion-forming units and sera were collected 35 days post-inoculation (134). Sera were assayed at 1:50. The background-corrected OD_{450} values of all ten mice inoculated with *C. trachomatis* L2 P^- 25667R (mice no. 2D1-2D10) ranged from -0.047-0.143 across all antigens. Two mice from the group inoculated with the same isolate transformed with pGFP::SW2 reacted with GST-PGP3 SW2 and PGP3 SW2. The OD_{450} results of these mice are summarised in Table 5.6. The OD_{450} values of all other mice from the same group ranged from -0.091-0.061 across all antigens. Mice no. ID7 and ID8 were also assayed against GST-PGP3 SW2 and GST in a western blot (5.2.4.2). 1 µg aliquots of recombinant protein were denatured in 1x SDS loading buffer or left untreated (2.10.2). 10 µl aliquots were analysed by SDS-PAGE, blotted with mice sera at 1:100 and visualised using an ECL kit, as described (2.10.8). No reaction to the GST negative control antigen was observed (lane 3, panels (b)-(c), Figure 5.6). A weak reaction to the GST-PGP3 SW2 trimer was observed using sera from mouse no. ID7 (lane 2, panel (b), Figure 5.6). Serum from mouse no. ID8 also reacted with the trimer of GST-PGP3 SW2 (lane 2, panel (c), Figure 5.6). No reaction with the monomer of GST-PGP3 SW2 was observed from either serum sample (lane 1, panels (b)-(c), Figure 5.6).

Table 5.6. Details of mice sera with high OD_{450} value in PGP3 SW2 ELISAs. Sera were assayed at 1:50 against 500 ng/well recombinant GST-PGP3 derived from *C. trachomatis* pSW2, 500 ng/well PGP3 derived from *C. trachomatis* pSW2 and 250 ng/well GST. OD_{450} values shown are the mean absorbance of two duplicate background-corrected values.

Mouse no.	GST-PGP3 SW2 (4T1)	PGP3 SW2 (6P1)	GST (4T1)
ID7	1.31	1.06	-0.002
ID8	3.19	2.90	-0.091

Figure 5.6. Antibody recognition of PGP3 in a Western blot using sera from mice urogenitally infected with *C. trachomatis* L2 P^- pGFP::SW2. Recombinant GST-PGP3 derived from *C. trachomatis* pSW2 was denatured (**lane 1**: 2% SDS, 1.25% BME, heated to 100°C for 5 minutes) or untreated (**lane 2**), analysed by SDS-PAGE on a 12% SDS gel and transferred to PVDF membrane. GST was included as a negative control and was denatured as described (**lane 3**). Sera from mice with OD_{450} >0.652 in our GST-PGP3 SW2 ELISA were added at 1:100 and antibodies detected using goat anti-mouse IgG-HRP (1:2000) and n ECL kit. [Positive control: mouse monoclonal anti-GST antibody **(panel a)**; mouse no. ID7 **(panel b)** and ID8 **(panel c)**]



5.4 DISCUSSION

This chapter describes the validation of PGP3 ELISAs to detect anti-PGP3 antibodies in patient serum samples based on recombinant PGP3 derived from *C. trachomatis* pSW2 (serovar E) and pLGV440 (serovar L1). These ELISAs were validated using serum collected from GUM patients with a known *C. trachomatis* status, as diagnosed by NAAT.

5.4.1 Recombinant coating antigen performance

At 98% specificity, overall sensitivities were 68.18% for the GST-PGP3 SW2 ELISA and 66.36% for the GST-PGP3 LGV440 ELISA, a difference of 1.82%. Although our GST-PGP3 SW2 ELISA was 1.8% more sensitive than the GST-PGP3 LGV440 ELISA, no statistically significant differences were found between sensitivities. Wills *et al.* (118) evaluated their ELISA (based on recombinant PGP3 antigen derived from *C. trachomatis* pLGV440) using well-characterised sera from 356 patients of mixed-sex who tested positive for *C. trachomatis* by NAAT (serum collected one month after diagnosis) and sera from over 700 patients aged 2-13-years-old that were presumed negative for any past exposure to *C. trachomatis*. 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%. Interestingly, although not statistically significant, our GST-PGP3 ELISAs were on average 7.85% more sensitive for female samples than male samples. This correlates with findings by Wills *et al.* 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 (118). Previous studies have found that oestrogen *in vitro* enhances the attachment of *C. trachomatis* to human endometrial epithelial cells (186) and a higher chlamydial burden is associated with increased levels of anti-*C. trachomatis* antibodies (187). This may account for the higher sensitivities seen in ELISAs using serum samples from females.

At least seven serological assays (75, 118, 122, 160, 163, 167, 168) using PGP3 have been described and two have reported sensitivities and specificities as high as 95.1%

(163) and 100% (168), 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 (118, 122, 163, 167), presence of symptoms (122, 168) or isolation of chlamydia by culture (160). Bas *et al.* (122) 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 (75, 168). 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 GUM patients with a 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. These serum samples were collected from patients at the time of their *C. trachomatis* diagnosis by NAAT and therefore some patients diagnosed as positive for *C. trachomatis* may not have seroconverted at the time of serum collection.

To investigate the possibility that the N-terminal GST fusion tag was lowering the sensitivity of our assay and to allow comparison with the ELISA described by Wills *et al.* (118), we repeated the ELISA using the same serum samples and protocol but instead used recombinant PGP3 derived from *C. trachomatis* pSW2 that had undergone enzymatic cleavage from the GST tag. This was to ensure that PGP3 alone would saturate the assay, as it was discovered that increasing the antigen coating concentration resulted in higher OD_{450} values obtained from assaying sera from patients diagnosed positive for *C. trachomatis* by NAAT, as discussed in chapter four. As the ELISA based on recombinant PGP3 antigen derived from *C. trachomatis* pSW2 was more sensitive than using the PGP3 LGV440 antigen, all human sera were assayed against the cleaved PGP3 derived from *C. trachomatis* pSW2 (PGP3 SW2) to compare sensitivity and specificity with that of the GST-tagged PGP3 SW2 indirect ELISA. Surprisingly, the specificity of the PGP3 SW2 ELISA was lower than that obtained from the GST-PGP3

SW2 ELISA. However, as discussed, our negative control serum samples were taken from patients with a negative diagnosis for *C. trachomatis* (by NAAT). Therefore, 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. The specificity of our PGP3 SW2 ELISA may have nevertheless been improved; higher OD_{450} values returned from patients with a negative *C. trachomatis* diagnosis by NAAT may have reflected the presence of low levels of anti-PGP3 antibodies within the samples from the sexually active patients. However, Western blot assays using serum samples that returned OD_{450} values above the GST-PGP3 SW2 ELISA OD_{450} cut-off revealed no reaction to monomeric or trimeric PGP3. It may have been beneficial to use negative control serum samples characterised by a combination of methods including NAAT.

5.4.2 Primary antibody response to C. trachomatis in mice

It is not possible to study the primary antibody response to *C. trachomatis* infection in humans by controlled experimentation. Therefore, informative infection experiments must be conducted in model systems. Ramsey *et al.* (135) infected outbred female Swiss Webster mice with either a naturally occurring plasmid-free isolate of *C. trachomatis* from the L2 serovar (L2 P^- 25667R) or the same isolate transformed with a recombinant chlamydial plasmid, pGFP::SW2, from serovar E. To investigate whether a chlamydial genital tract infection can result in the production of anti-PGP3 antibodies detectable in an ELISA, sera collected from mice urogenitally infected with *C. trachomatis* were assayed against recombinant PGP3 in the GST-PGP3 SW2 and PGP3 SW2 ELISAs. Two mice reacted with both GST-PGP3 SW2 and PGP3 SW2. These mice were also assayed against GST-PGP3 SW2 and GST in a Western blot assay and reactions to the GST-PGP3 SW2 trimer were observed. As serum samples were collected 35 days post-inoculation, this may explain why only two out of ten mice reacted with recombinant PGP3 in an ELISA and Western blot assay, as these mice may have not had enough time to seroconvert. These serum samples were collected from mice in a separate experiment and therefore we had no control when serum samples would be collected post-inoculation (135). Despite this, this validates our PGP3 ELISAs and supports the phenomenon that positive responses produced from sera collected from *C. trachomatis* positive patients are due to the presence of anti-PGP3 antibodies. Sera from mice urogenitally infected with *C. muridarum* have been western blotted against PGP3 in a

previous study (155). Interestingly, sera from mice in our study only reacted with the trimeric conformation of GST-PGP3 which agrees with previous reports that human antibody recognition is dependent on the trimeric conformation of PGP3 (124, 125). However, it has also been reported that reaction to the trimer and/or monomer of PGP3 is dependent on the mouse strain used (155).

5.4.3 Controlling inter- and intra- assay variation

As our GST-PGP3 SW2 ELISA uses PGP3 derived from a common genital tract strain of *C. trachomatis* (pSW2, serovar E), and because the GST-PGP3 SW2 ELISA had a higher sensitivity than the LGV440 ELISA, it was concluded that this ELISA would be the most suitable for seroepidemiological analyses and to track urogenital *C. trachomatis* infections. Therefore, the intra- and inter-assay variation of the GST-PGP3 SW2 ELISA was analysed. For these calculations, six sera that were negative and positive for anti-PGP3 antibodies, as determined from previous ELISAs and Western blotting experiments, were selected. 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 (CV) for each control were calculated and 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. Some difficulty was encountered in producing a protocol to demonstrate the reproducibility of our assay as there are no 'gold-standard' guidelines to calculate the inter- and intra-assay reproducibility of a new serological assay. Furthermore, only Wills *et al.* (2009) had demonstrated the reproducibility of their PGP3 ELISA. They assayed five serum samples with an OD_{450} range between 0-3.5 against their PGP3 LGV440 antigen using twenty independent assays to calculate standard deviation and intra- and inter-assay variation. However, the reasons for how or why these numbers of samples and assays were selected were not stated. For our ELISAs, advice was sought from the Seroepidemiology Unit at Public Health England for guidelines on how to conduct these experiments. Three patient sera from patients known to react to GST-PGP3 derived from *C. trachomatis* pSW2 were selected as positive controls, as determined in previous ELISAs. These sera were selected based on their OD_{450} range between ~0.7-3.3, noting the cut-off of OD_{450} 0.652 for the GST-PGP3 SW2 ELISA. We also selected three sera

known not to react with GST-PGP3 SW2 in an ELISA as negative controls and these were selected based on their mean OD_{450} range between ~0.1-0.4. Mouse monoclonal anti-GST and rabbit polyclonal anti-PGP3 positive control antibodies were also included for a total of eight control samples. Three other operators were used to conduct these experiments, all of which were independent to the project and one operator that was independent to our research group. Operators were also blinded to the serum and positive control antibodies. This is the most robust method described to demonstrate the reproducibility of a PGP3 ELISA. Although the experiments were conducted taking the advice from the SEU into consideration, concerns were raised about the potential accuracy of using correlation of variation (CV) and standard deviation (SD) to calculate intra- and inter-assay variation. It was noted that the higher the mean OD_{450} value obtained from a serum sample in an ELISA, the lower the CV and higher the SD are more likely to occur. A lower CV would suggest a more accurate and reproducible serological assay. However, as CV is calculated using the mean percentage difference from the total mean OD_{450} value of a sample, a control with a high OD_{450} value but more variation between assay runs is likely to return a lower CV in comparison with a low OD_{450} value control sample with little variation between assay runs. Therefore, when analysing CV and SD values of serological assays, it is important to consider the number of controls, raw data and methods used to calculate those values as carefully-selected control samples may produce more desirable intra- and inter-assay variation results. Despite these concerns, there are currently no other recommended or accurate methods to demonstrate assay reproducibility.

5.4.1 Conclusions

- Recombinant GST-PGP3 derived from a urogenital strain of *C. trachomatis*, serovar E (pSW2) is the optimal ELISA format to detect anti-PGP3 antibodies in patient serum.
- GST expressed and purified using the same protocol as GST-PGP3 is a suitable control to assess potential false-positive background from sera in our PGP3 ELISAs.
- The ELISA with GST-PGP3 from SW2 exceeds the overall sensitivity (68.18% vs 57.9%) and specificity (98.0% vs 97.6%) of the L1 PGP3 ELISA reported by Wills *et al.* (118), as well as reproducibility, and is ready to be used in seroprevalence studies.

CHAPTER SIX: SEROPREVALENCE OF ANTIBODIES TO PGP3 IN 13-15-YEAR-OLD CHILDREN IN ENGLAND

6.1 INTRODUCTION

6.1 Seroprevalence of C. trachomatis antibodies in 13-15-year-olds in England

No systematic investigation of the seroprevalence of *C. trachomatis* in the under 16s population in England has been conducted, despite evidence of chlamydial diagnoses and increasingly earlier sexual debut. In 2013, over 21,000 young people under 15 in England were screened for chlamydial infection by NAATs (89). 1,100 (5.4%) of these tests were diagnosed as positive, representing 0.2% of the overall population for young people under 15. As this age group is not actively recruited by the NCSP for chlamydial screening, prevalence of *C. trachomatis* is likely higher. Understanding the seroprevalence of chlamydial infection is essential as this would provide evidence of a problem to be addressed by targeted intervention and screening.

The NCSP justification for the cut-off at age 15 for chlamydial screening is unclear. The NCSP opportunistically screens 16-24-year-olds. However, 15-year-olds are often only tested under special circumstances and parental notification is often encouraged in accordance with the Fraser Guidelines (109). In 2011, an English survey reported that 1.9% experienced non-consensual intercourse and 8.3% experienced some form of sexual abuse before age 16 (178). Another study found that 29.9% of men and 25.6% of women reported having their first sexual experience before age 16 (176). Over 14% of respondents reported their first sexual intercourse experience at age 13-14-years-old. The same study reported 17.9% of males and 21.7% of females who had their first sexual encounter at age 13-14-years-old reported a lack of any contraceptive use in comparison to 9.8% and 10% of 15-year-old males and females, respectively. Although no systematic seroprevalence study in England has been conducted, in 2009 Wills *et al.* (118) included sera collected from children in England aged 2-13-years-old as presumed negative controls for their PGP3 ELISA evaluation. Of the 747 sera assayed, 25 (3.34%) were found to be positive for *C. trachomatis* antibodies using microimmunofluorescence (MIF). In the USA, Goodhew *et al.* (167) also conducted a serological study using sera collected from a non-endemic cohort of children under 6-years-old as presumed negative controls to evaluate their PGP3 assay. Of the 122 sera assayed, 3 (2.4%) were positive for anti-PGP3 antibodies. In 2013, Horner *et al.* (165)

determined the overall PGP3 antibody prevalence in 17-18-year-old women in England to be 13.24%. However, an important gap in epidemiological data still exists for the under 16s.

The previous chapter described the evaluation of a sensitive and specific indirect ELISA using PGP3 derived from a urogenital strain of *C. trachomatis*, serovar E (GST-PGP3 SW2). Since the validation of this ELISA, a double-PGP3 antigen sandwich ELISA by Horner *et al.* (2016) (172) reporting a higher sensitivity for serum samples collected from females (82.9% vs 71.93%) was described. However, the GST-PGP3 SW2 ELISA is 9.75% more sensitive (54.4% vs 64.15%) for male samples. Furthermore, this ELISA did not include a negative control antigen whereas the GST-PGP3 SW2 ELISA allowed the use of recombinant GST as a negative control antigen to assess false-positive reactivity from the sera. In the current chapter, this GST-PGP3 SW2 ELISA was used to detect anti-PGP3 antibodies in a large cohort of serum samples collected from 13-15-year-olds sourced from various regions across England. Using this data, we sought to determine the seroprevalence of antibodies to PGP3 in 13-15-year-olds in England.

6.1.1 Aim

- To use the validated PGP3 ELISA to detect anti-PGP3 antibodies in sera collected from 13-15-year-old children and use the data to determine the seroprevalence of *C. trachomatis* in this population in England.

6.1.2 Objectives

- To use the PGP3 ELISA to assay all samples against recombinant GST-PGP3 (3.3.3) derived from *C. trachomatis* serovar E (pSW2).
- To re-assay all serum samples that return an OD result on the borderline or above the cut-off against recombinant GST-PGP3 SW2.
    - Assay all serum samples that return an OD result on the borderline or above the cut-off against recombinant GST as a negative control to exclude the possibility of cross-reactivity to the GST tag from the sera.
- To use well-characterised serum and antibody controls to confirm results and monitor assay reproducibility.

- To determine which serum samples are positive for anti-PGP3 antibodies and use the data to determine the seroprevalence of *C. trachomatis* within this population.

6.2 METHODS

6.2.1 Control antibodies and patient serum

Three patient sera were selected as positive controls and three patient sera were selected as negative controls, as determined in previous ELISAs and Western blots (5.2.6.1). Mouse monoclonal anti-GST IgG (1:10,000) (Invitrogen, Life Technologies) and rabbit polyclonal anti-PGP3 (1:10,000) antibodies were used as positive controls. All control antibodies were assayed in duplicate.

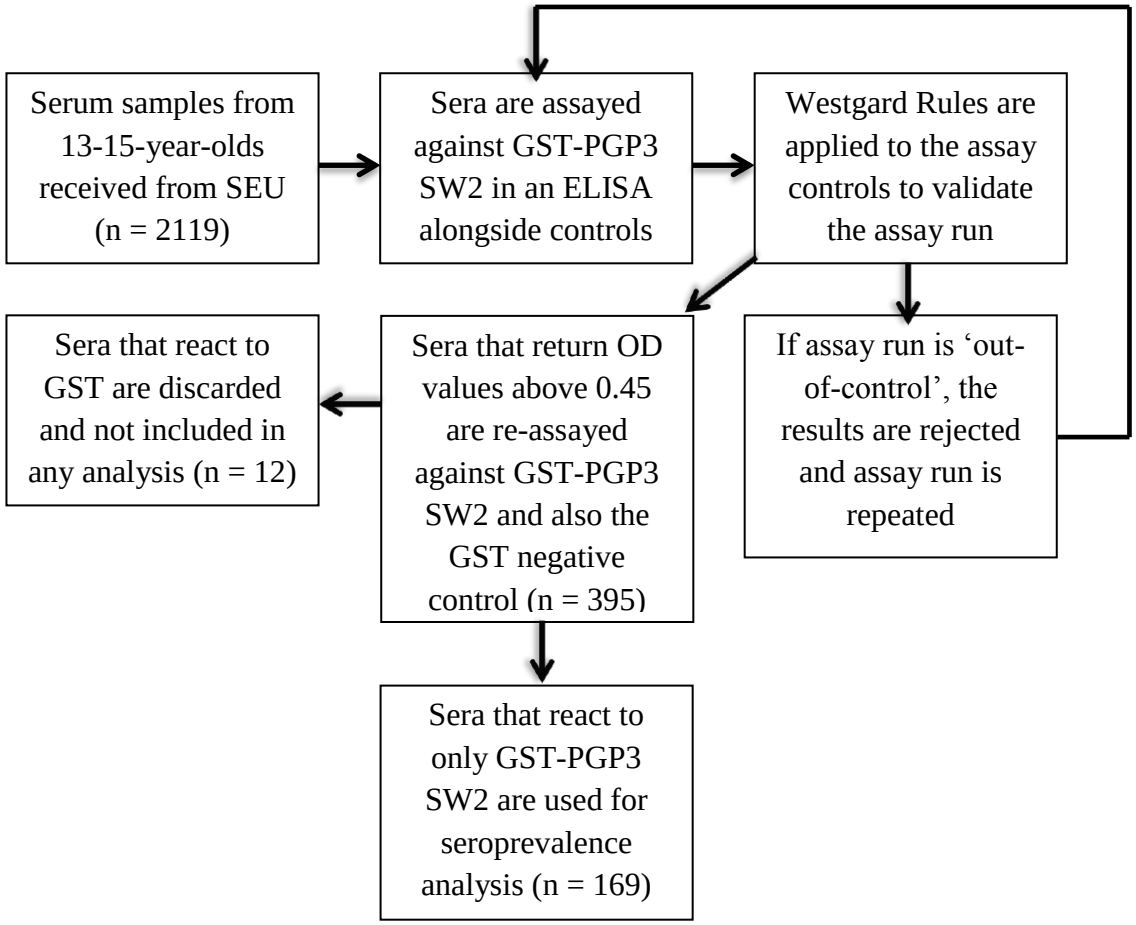
6.2.2 Serum samples

2119 anonymised and unlinked serum samples were received from the Seroepidemiology Unit at PHE (Manchester, UK). The number of serum samples required to reach statistical significance from 15-year-olds was calculated based on the 3.3% seroprevalence rate of 2-13-year-olds as reported by Wills *et al.* (118) and was calculated by Mr Scott Harris (statistician, R&D support at the University of Southampton). The number of serum samples collected from 13- and 14-year-olds was calculated based on the findings of antibody seroprevalence in 15-year-olds and interviewee responses from a sexual behaviour survey by Wellings *et al.* (176). Samples were shipped on dry ice and stored at -80°C until use. Samples were collected from 1062 male and 1057 female 13-year-old (females = 350; males = 354), 14-year-old (females = 353; males = 354) and 15-year-old (females = 354; males = 354) patients across England between 2009-2014. 193 of the samples were sourced from GUM clinics, 547 samples were not sourced from GUM clinics and 1379 samples were from an unknown/unrecorded source. The only known details of the patient serum samples were patient sex, year of serum collection, serum source (GUM/non-GUM/unknown) and source location (city/region only, not postcode).

6.2.3 Indirect ELISA using sera from 13-15-year-olds

Patient sera were blinded and assayed against GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3) using the method described in 5.2.3. Control antibodies were assayed in duplicate and background was corrected using the method described in 4.3.2. Samples that returned a background-corrected OD_{450} result above 0.450 were re-assayed against GST-PGP3 SW2 in duplicate and the mean OD result was calculated (Figure 6.1). 0.450 is the lower quartile (LQ) of OD values obtained from patients diagnosed positive for *C. trachomatis* by NAAT in the GST-PGP3 SW2 ELISA (5.3.1) and was chosen to include patient samples with OD values just below the 0.652 cut-off. These samples were also assayed against GST as a negative control using the method described in 5.2.3. Serum samples with a mean background-corrected OD value above 0.652 against GST-PGP3 SW2 were considered positive for anti-PGP3 antibodies (5.3.1.3).

Figure 6.1 Strategy for assaying serum samples collected from 13-15-year-olds in England against GST-PGP3 derived from *C. trachomatis* pSW2 in an indirect ELISA.



6.2.3.1 Westgard Rules

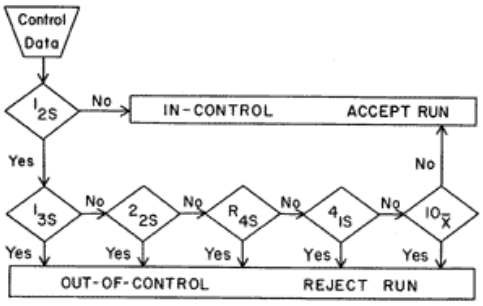
Multirule quality control Westgard rules were used to determine if any repeat experiments (assay runs) were required on some serum sets (188). These rules are used as an indication of whether a run of an assay is 'in-control' or 'out-of-control'. Three upper and lower OD value control limits (+/-1, 2, 3_S) were calculated and individual control results were displayed on Levey-Jennings charts. Means and standard deviations were calculated using OD_{450} data from twenty independent assays using four operators (5.3.3). The equation for the upper and lower control limit calculations and multirule quality control Westgard rules are listed below and in Figure 6.2.

Control limits: Mean OD_{450} +/- (SD*control limit)

Multirule Quality Control Westgard Rules

- **1_{3S} –** the assay run results are rejected when any control reading exceeds the mean +/- the 3_S control limit
- **1_{2S} –** does not indicate automatic rejection of results but requires inspection of all controls when any control exceeds the mean +/- the 2_S control limit
- **2_{2S} –** the assay run results are rejected when two consecutive control results exceed the mean +/- the 2_S control limit
- **R_{4S} –** the assay run results are rejected when one control result exceeds the mean + 2_S and another control result exceeds the mean -2_S control limit
- **4_{1S} –** the assay run results are rejected when four consecutive control results exceed the same mean +/- 1_S control limit
- **10_X –** the assay run results are rejected when ten consecutive control results are either all + or – the mean

Figure 6.2 Summary of the multirule quality control Westgard rule process. Assay run results are rejected when the results are out-of-control and do not adhere to the multirule QC.



6.2.4 Detection of recombinant PGP3 in a Western blot using sera from 13-15 year-olds

Detection of monomeric and trimeric GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3) and GST using patient sera and mouse monoclonal anti-GST and rabbit polyclonal anti-PGP3 control antibodies (6.2.1) was conducted using the method described in 5.2.3.2.

6.3 RESULTS

To determine the seroprevalence of anti-PGP3 antibodies in 13-15-year-olds, serum samples were collected from males and females aged 13-15-year-olds (6.2.2) and samples were blinded and assayed at 1:100 diluted in blocking buffer against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 in the indirect ELISA (5.2.3, 6.2.3). To determine antibody prevalence, serum samples were then unblinded and grouped according to their PGP3 antibody positivity, sex, age, source of sample collection, location of collection and/or year of collection (6.3.1-6.3.3).

6.3.1 Overall seroprevalence of anti-PGP3 antibodies in 13-15-year-olds from England

To determine the overall anti-PGP3 antibody prevalence in serum samples collected from 13-15-year-olds, samples were unblinded and grouped according to their anti-PGP3 antibody positivity, patient sex and serum source (Table 6.1 and Figure 6.3). Of the 2107 serum samples assayed in the GST-PGP3 SW2 ELISA, a total of 169 samples returned a mean background-corrected OD_{450} value above 0.652 (females) or 0.655 (males) and were positive for anti-PGP3 antibodies (8.02%) (appendix VI). A total of 12 samples were considered positive for anti-GST antibodies: 5 samples from male 13-year-olds, 3 from female 14-year-olds, 3 from male 14-year-olds, and 1 from a male 15-year-old. These were not included in any statistical analyses (final serum total = 2107) (6.3.4.1).

Of the total 699 serum samples collected from male and female 13-year-olds (Table 6.1A), 48 serum samples (6.87%) were positive for anti-PGP3 antibodies. The overall antibody prevalence in serum samples sourced from GUM clinics was 5/27 (18.52%). Antibody prevalence in serum samples collected from male 13-year-olds sourced from GUM clinics was 25.00%. However, only 8 serum samples were assayed. The overall antibody prevalence in serum samples not sourced from GUM clinics was 9/193 (4.66%) and prevalence in samples from unknown sources was 34/479 (7.10%). Overall antibody prevalence in female serum samples collected from non-GUM sources and unknown sources was higher than the antibody prevalence found in male samples from the same serum sources (6.67% vs 2.91% and 8.30% vs 5.88%, respectively).

A total of 47/701 (6.70%) collected from male and female 14-year-olds were positive for anti-PGP3 antibodies (Table 6.1B). The overall antibody prevalence in serum samples sourced from GUM clinics was 10/55 (18.18%). However, only 12 of these serum samples were collected from males and none were found to be positive for anti-PGP3 antibodies. The overall antibody prevalence in serum samples not sourced from GUM clinics was 8/183 (4.37%) and prevalence in samples from unknown sources was 29/463 (6.26%), 0.29% and 0.84% lower than the percentage prevalence found in the same serum groups from 13-year-olds, respectively. However, antibody prevalence in male serum samples sourced from non-GUM clinics was 2.91% higher in 14-year-olds (6/103 (5.82%)).

Of the total 707 serum samples collected from male and female 15-year-olds (Table 6.1C), 74 serum samples (10.47%) were positive for anti-PGP3 antibodies. Prevalence of antibodies in serum samples sourced from GUM clinics was higher in samples collected from females in comparison to samples collected from males (28.35% vs 11.54%, respectively). However, of the total 111 samples sourced from GUM clinics, only 26 were collected from males. In contrast to the findings in the 13- and 14-year-old serum groups, prevalence of antibodies in serum samples collected from both male and female non-GUM sources were higher than the prevalence rates found in serum samples from unknown sources (11.00% vs 5.73% and 10.45% vs 7.92%, respectively).

GraphPad Prism version 6.0 was used to calculate the two-tailed Fisher's exact test and 95% confidence intervals (Table 6.1 and 6.2). Results were considered to be statistically significant if $p = <0.05$. Fisher's exact test was used to test for differences in antibody prevalence between patient sexes (Table 6.1). No association was found (p values = 0.096-1.000). The Fisher's exact test was also performed to test for differences in antibody prevalence between patient age groups (Table 6.2). Although there was a notable increase in the antibody prevalence in female serum samples sourced from GUM clinics between ages 13 to 15 at the time of serum collection (15.79% to 28.23%; Table 6.1), no statistically significant differences in prevalence was found. There are also notable differences in antibody prevalence in serum samples collected from female 13-, 14- and 15-year-olds sourced from non-GUM clinics (6.67%, 2.50% and 10.45%, respectively). However, no statistically significant differences in prevalence were found. The only statistically significant difference in seroprevalence rates was found

between serum groups collected from non-GUM clinic sources from 13- (3/103 (2.91%)) and 15-year-old male patients (11/100 (11.00%)) (p value = 0.0274**).

Table 6.1. Seroprevalence of anti-PGP3 antibodies in 13-15-year-olds in England by age group and source of serum collection. Table shows the seroprevalence of antibodies to PGP3 in serum samples collected from 2107 patients aged 13-15-years-old at the time of serum collection. GraphPad Prism version 6.0 was used to calculate two-tailed Fisher's exact test and results were considered to be statistically significant if p = <0.05. *The Fisher's exact was also performed to test for differences in antibody prevalence between patient age groups (Table 6.2). **[A]** 13-year-olds; **[B]** 14-year-olds; **[C]** 15-year-olds

A

Source of Sera	Antibody Prevalence in 13-year-olds % (95% CI)			Difference in female and male prevalence? (P value)
	Overall (Female & Male)	Female	Male	
GUM Clinic n = 27	5/27 **18.52%** (6.30-38.08%)	3/19 **15.79%** (3.38-39.58%)	2/8 **25.00%** (3.18-65.09%)	0.616
Non-GUM n = 193	9/193 **4.66%** (2.15-8.67%)	6/90 **6.67%** (2.49-13.95%)	3/103* **2.91%** (0.61-8.28%)	0.308
Not Known n = 479	34/479 **7.10%** (4.97-9.78%)	20/241 **8.30%** (5.14-12.53%)	14/238 **5.88%** (3.25-9.67%)	0.374

B

Source of Sera	Antibody Prevalence in 14-year-olds % (95% CI)			Difference in female and male prevalence? (P value)
	Overall (Female & Male)	Female	Male	
GUM Clinic n = 55	10/55 **18.18%** (9.08-30.91%)	10/43 **23.26%** (11.76-38.63%)	0/12 **0%** (0.00-26.47%)	0.096
Non-GUM n = 183	8/183 **4.37%** (1.91-8.43%)	2/80 **2.50%** (0.30-8.74%)	6/103 **5.82%** (2.17-12.25%)	0.496
Not Known n = 463	29/463 **6.26%** (4.24-8.87%)	18/227 **7.93%** (4.77-12.24%)	11/236 **4.66%** (2.35-8.19%)	0.180

C

Source of Sera	Antibody Prevalence in 15-year-olds % (95% CI)			Difference in female and male prevalence? (P value)
	Overall (Female & Male)	Female	Male	
GUM Clinic n = 111	27/111 **24.32%** (16.68-33.38%)	24/85 **28.23%** (19.00-39.04%)	3/26 **11.54%** (2.45-30.15%)	0.117
Non-GUM n = 167	18/167 **10.78%** (6.51-16.50%)	7/67 **10.45%** (4.30-20.35%)	11/100* **11.00%** (5.62-18.83%)	1.000
Not Known n = 429	29/429 **6.75%** (4.57-9.56%)	16/202 **7.92%** (4.60-12.54%)	13/227 **5.73%** (3.08-9.59%)	0.442

Table 6.2. Comparison of anti-PGP3 antibody seroprevalence rates between 13-15-year-old age groups using the Fisher's exact test. GraphPad Prism version 6.0 was used to calculate the Fisher's exact test and results were considered to be statistically significant if p = <0.05. A statistically significant difference in seroprevalence rates was found between serum groups collected from 13- and 15-year-old male patients from non-GUM sources (*p value = 0.0274).

Serum Group		Fisher's Exact Test P Value			
		Overall P Value	**Age 13 vs 14**	**Age 13 vs 15**	**Age 14 vs 15**
GUM	**Females**	0.497	0.737	0.387	0.672
	Males	0.210	0.147	0.570	0.538
Non-GUM	**Females**	0.141	0.284	0.560	0.080
	Males	0.062	0.498	**0.0274***	0.212
Not Known	**Females**	0.986	1.000	1.000	1.000
	Males	0.816	0.687	1.000	0.678

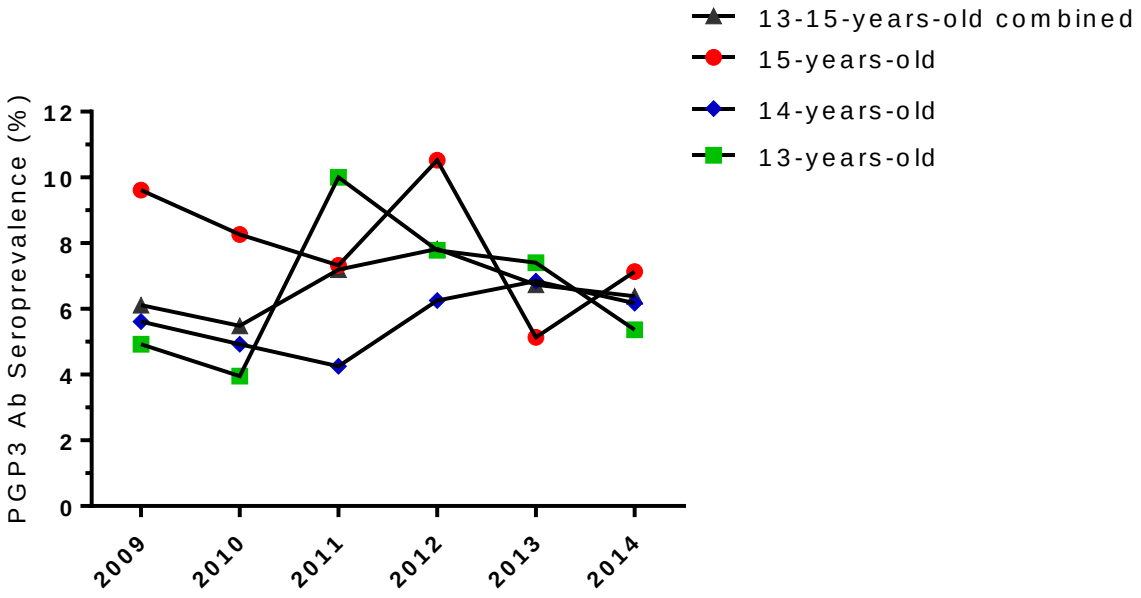
6.3.2 Seroprevalence of anti-PGP3 antibodies by year of sample collection

To investigate a potential association between anti-PGP3 antibody positivity in serum samples and the year of serum sample collection, serum samples were unblinded and grouped according to their PGP3 antibody positivity, patient age at the time of serum collection and year of serum collection (Table 6.3 and Figure 6.3). For analysis, male and female serum samples sourced from non-GUM clinics and unknown sources were combined. Samples sourced from GUM clinics were excluded. A total of 1914 serum samples collected from 13-15-year-olds at the time of serum collection were collected from 2009-2014. GraphPad Prism 6.0 was used to calculate the Chi-square test for trend and results were considered to be statistically significant if $p = <0.05$. No statistical significance in association was found between year of sample collection and overall antibody prevalence in serum samples from combined age groups ($p = 0.723$). Furthermore, no statistical significance in association was found between year of sample collection and the antibody prevalence in serum samples from 13-, 14- and 15-year-olds (p values = 0.335, 0.539, 0.421, respectively). Although not statistically significant, there is a noticeable increase in seroprevalence of 3.95% to 10.00% from 2010 to 2011, respectively, in serum samples collected from 13-year-olds. Similarly, there is a noticeable decrease in seroprevalence of 10.53% to 5.13% from 2012 to 2013, respectively, in serum samples collected from 15-year-olds. However, only 57 samples were collected from 15-year-olds in 2012 and the total number of serum samples collected from 2009 to 2014 from each age group ranged from 57 to 150.

Table 6.3. Seroprevalence of anti-PGP3 antibodies in 13-15 year-olds in England by age group and year of sample collection. Table shows the combined anti-PGP3 antibody seroprevalence of 1914 serum samples collected from male and female 13-15-year-olds, samples not sourced from GUM clinics and those from unknown sources. Samples known to be sourced from GUM clinics were excluded. Chi-square for trend was calculated by GraphPad Prism version 6.0 and results were considered to be statistically significant if p = <0.05.

Age (Years)	Year of Serum Collection	Total Tested	PGP3 Antibody Prevalence (%)	95% CI	Chi-Square for Trend (P Value)
13	2009	122	4.92	1.83-10.40	0.335
	2010	152	3.95	1.46-8.39	
	2011	90	10.00	4.68-18.14	
	2012	90	7.78	3.18-15.37	
	2013	162	7.41	3.89-12.58	
	2014	56	5.36	1.12-14.87	
14	2009	107	5.61	2.09-11.81	0.539
	2010	122	4.92	1.83-10.40	
	2011	94	4.25	1.17-10.54	
	2012	96	6.25	2.33-13.12	
	2013	146	6.85	3.33-12.24	
	2014	81	6.17	2.03-13.82	
15	2009	104	9.62	4.71-16.97	0.421
	2010	109	8.26	3.85-15.10	
	2011	150	7.33	3.72-12.74	
	2012	57	10.53	3.96-21.52	
	2013	78	5.13	1.42-12.61	
	2014	98	7.14	2.92-14.17	
13-15 combined age groups	2009	333	6.11	4.19-9.83	0.723
	2010	383	5.48	3.43-8.26	
	2011	334	7.19	4.66-10.50	
	2012	243	7.82	4.77-11.94	
	2013	386	6.74	4.45-9.71	
	2014	235	6.38	3.62-10.31	

Figure 6.3. Seroprevalence of anti-PGP3 antibodies in 13-15 year-olds in England by age group and year of sample collection. Graph shows the combined anti-PGP3 antibody seroprevalence of 1914 serum samples collected from male and female 13-15-year-olds, samples not sourced from GUM clinics and those from unknown sources. Samples known to be sourced from GUM clinics were excluded.



6.3.3 Seroprevalence of anti-PGP3 antibodies by region and city

To investigate a potential association between anti-PGP3 antibody positivity in serum samples and the region of serum collection, samples were unblinded and grouped according to their PGP3 antibody positivity, source of serum collection (GUM clinic/non-GUM/unknown) and the region of serum collection (Table 6.4 and Figure 6.4). For analysis, male and female serum samples collected from all age groups were combined. A total of 2107 serum samples collected from 13-15-year-olds at the time of serum collection were included in the analysis. GraphPad Prism 6.0 was used to calculate the Chi-square test for association and results were considered to be statistically significant is $p = <0.05$.

Overall, no association was found between the region of serum collection and antibody prevalence in serum samples (p values = 0.062-0.847; Table 6.4A). Similarly, no association was found between the city of serum collection and antibody prevalence in serum samples (p values = 0.137-0.733; Table 6.4B). The highest antibody prevalence found in serum samples sourced from GUM clinics were collected from the London region (31.15%; Table 6.4A). Although a total of 770 serum samples were sourced from the North West region (Table 6.4A), only one GUM clinic sample was collected. Analysis of serum samples from combined non-GUM and unknown sources revealed that the North East region had the lowest antibody prevalence of 4/111 (3.60%) and the West Midlands had the highest antibody prevalence of 6/65 (9.23%) (Table 6.4A and Figure 6.4A). The total number of serum samples collected from each region from each serum source ranged from 0 to 405. Further analysis of serum samples from combined non-GUM and unknown sources revealed that samples collected from St. George's Hospital (London) had the lowest antibody prevalence of 0/49 (0%) and samples collected from Barts and the London School of Medicine and Denistry had the highest antibody prevalence of 5/20 (25.00%) (Table 6.4B and Figure 6.4B).

Table 6.4. Seroprevalence of anti-PGP3 antibodies in 13-15 year-olds in England by region and source of serum collection. Combined anti-PGP3 antibody seroprevalence of serum samples collected from male and female 13-15-year-olds. *Chi-square for association was calculated by GraphPad version 6.0 and results were considered to be statistically significant if p = <0.05. N/A = Not applicable. (Barts Hospital: Barts and the London School of Medicine and Denistry; PHL London: Public Health Laboratory London; St. George's Hospital: St. George's University Hospitals, London). **[A]** Seroprevalence by region; **[B]** Seroprevalence by city/town/hospital

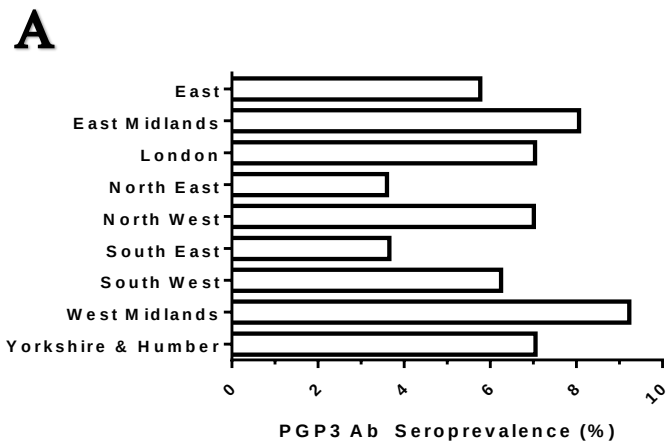
A

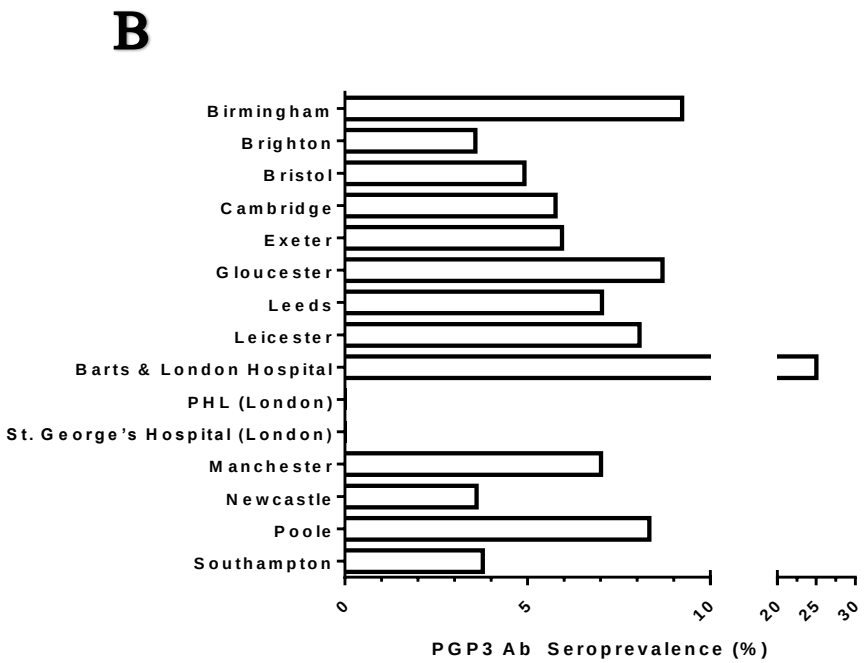
Region	Antibody Prevalence % (95% CI)			
	GUM	**Non-GUM**	**Not Known**	**Non-GUM/NK Combined**
East	2/14 **14.29%** (1.78-42.81%)	1/2 **50%** (1.26-98.74%)	2/50 **4.00%** (0.49-13.71%)	3/52 **5.77%** (1.21-15.95%)
East Midlands	4/19 **21.05%** (6.05-45.57%)	1/6 **16.67%** (0.42-64.12%)	4/56 **7.13%** (1.98-17.29%)	5/62 **8.07%** (2.67-17.83%)
London	14/41 **31.15%** (20.08-50.60%)	0/39 **0%** (0.00-9.03%)	5/32 **15.63%** (5.28-32.79%)	5/71 **7.04%** (2.33-15.67%)
North East	14/60 **23.33%** (13.38-36.04%)	3/47 **6.38%** (1.34-17.54%)	1/64 **1.56%** (0.04-8.04%)	4/111 **3.60%** (0.89-8.97%)
North West	0/1 **0%** (0.00-97.50%)	27/365 **7.40%** (4.93-10.58%)	27/405 **6.67%** (4.44-9.55%)	54/770 **7.01%** (5.31-9.05%)
South East	1/5 **20.00%** (0.50-71.64%)	0/12 **0%** (0.00-26.47%)	3/69 **4.35%** (0.91-12.19%)	3/81 **3.70%** (0.77-10.44%)
South West	7/53 **13.21%** (5.48-25.34%)	3/72 **4.17%** (0.87-11.70%)	16/232 **6.90%** (4.00-10.96%)	19/304 **6.25%** (3.80-9.59%)
West Midlands	0/0 N/A N/A	0/0 N/A N/A	6/65 **9.23%** (3.46-19.02%)	6/65 **9.23%** (3.46-19.02%)
Yorkshire and Humber	0/0 N/A N/A	0/0 N/A N/A	28/398 **7.03%** (4.73-10.01%)	28/398 **7.04%** (4.73-10.01%)
P value*	**0.339**	**0.062**	**0.365**	**0.847**

B

Town/City	Antibody Prevalence % (95% CI)			
	GUM	**Non-GUM**	**Not Known**	**Non-GUM/NK Combined**
Birmingham	0/0 N/A N/A	0/0 N/A N/A	6/65 **9.23%** (3.46-19.02%)	6/65 **9.23%** (3.46-19.02%)
Brighton	0/1 **0%** (0.00-97.50%)	0/0 N/A N/A	1/28 **3.57%** (0.10-18.35%)	1/28 **3.57%** (0.10-18.35%)
Bristol	0/3 **0%** (0.00-70.76%)	0/9 **0%** (0.00-33.63%)	3/52 **5.77%** (1.21-15.95%)	3/61 **4.92%** (1.03-13.71%)
Cambridge	2/14 **14.29%** (1.78-42.81%)	1/2 **50.00%** (1.26-98.74%)	2/50 **4.00%** (0.49-13.71%)	3/52 **5.77%** (1.21-15.95%)
Exeter	6/48 **12.50%** (1.26-98.74%)	3/63 **4.76%** (0.99-13.29%)	8/122 **6.56%** (2.87-12.51%)	11/185 **5.95%** (3.01-10.39%)
Gloucester	1/2 **50%** (1.26-98.24%)	0/0 N/A N/A	4/46 **8.70%** (2.42-20.80%)	4/46 **8.70%** (2.42-20.80%)
Leeds	0/0 N/A N/A	0/0 N/A N/A	28/398 **7.04%** (4.73-10.01%)	28/398 **7.04%** (4.73-10.01%)
Leicester	4/19 **21.05%** (6.05-45.56%)	1/6 **16.67%** (0.42-64.12%)	4/56 **7.14%** (1.99-17.30%)	5/62 **9.62%** (3.20-21.03%)
Barts &London Hospital	2/9 **22.22%** (2.81-60.01%)	0/0 N/A N/A	5/20 **25.00%** (8.66-49.12%)	5/20 **25.00%** (8.66-49.12%)
PHL London	2/4 50.00% (6.76-93.24%)	0/2 0% (0.00-84.19%)	0/0 N/A N/A	0/2 **0%** (0.00-84.19%)
St. George's Hospital (London)	10/28 **35.71%** (18.64-55.94%)	0/37 **0%** (0.00-9.49%	0/12 **0%** (0.00-26.47%)	0/49 **0%** (0.00-7.25%)
Manchester	0/1 **0%** (0.00-97.5%)	27/365 **7.40%** (8.93-10.58%)	27/405 **6.67%** (4.44-9.55%)	54/770 **7.01%** (5.31-9.05%)
Newcastle	14/60 **23.33%** (13.38-36.04%)	3/47 **6.38%** (1.37-17.54%)	1/64 **1.56%** (0.04-81.40%)	4/111 **3.60%** (0.99-8.97%)
Poole	0/0 N/A N/A	0/0 N/A N/A	1/12 **8.33%** (0.21-38.48%)	1/12 **8.33%** (0.21-38.48%)
Southampton	1/4 **25.00%** (0.63-80.59%)	0/12 **0%** (0.00-26.47%)	2/41 **4.88%** (0.60-16.53%)	2/53 **3.77%** (0.46-12.98%)
P value*	**0.733**	**0.581**	**0.274**	**0.137**

Figure 6.4. Seroprevalence of anti-PGP3 antibodies in 13-15-year-olds in England by region. Graphs show combined anti-PGP3 antibody seroprevalence rates of serum samples collected from female and male 13-15-year-olds from combined unknown and non-GUM clinic serum sources. Serum samples sourced from GUM clinics were excluded. Chi-square for association was calculated by GraphPad Prism version 6.0 and results were considered to be statistically significant if p = <0.05. **[A]** Seroprevalence by region (p = 0.847); **[B]** Seroprevalence by city/town/hospital (p = 0.137).





6.3.4 Multirule quality control (QC) Westgard Rules and control limit

Westgard Rules were used to determine if any repeat screening experiments (assay runs) were required on some serum sets. Three upper and lower control limits were calculated using eight serum and positive control antibodies across twenty independent assays (5.3.3) using the equation = (mean OD_{450} +/- (SD*control limit)). Means and standard deviations were calculated using GraphPad Prism 6.0 using background-corrected OD_{450} values obtained from the mean duplicate values of two plates across twenty independent assays. Using Westgard Rules (6.2.3.1), OD control limits were used to determine if the OD values obtained from the control antibodies an assay run is 'in-control' or 'out-of-control'. Levey-Jennings charts were produced using GraphPad Prism 6.0 to determine if any assay runs conducted using the sera collected from 13-15-year-old children (6.2.2) required a repeated assay run (appendix V). A total of 32 assay runs were conducted to assay the 2119 serum samples against GST-PGP3 SW2 and GST in the indirect ELISA (6.2.3). Data from assays and repeated assays with validated 'in-control' runs were used for statistical analyses and seroprevalence calculations (6.3.1-6.3.3).

6.3.5 Detection of anti-PGP3 antibodies in sera collected from 13-15-year-olds using recombinant GST-PGP3 derived from C. trachomatis pSW2 in a Western blot

Western blots were conducted using example serum samples collected from 13-15-year-old patients to investigate serum samples that returned high, borderline and low background-corrected OD_{450} values when assayed against recombinant GST-PGP3 SW2 in the indirect ELISA (6.3.1). 1 μg aliquots of purified recombinant GST-PGP3 derived from *C. trachomatis* pSW2 (3.3.3.1) and GST (3.3.3.2) were denatured by heating in SDS loading buffer or left untreated (no heat or SDS) (2.10.2), analysed by SDS-PAGE on 12.5% SDS gels (2.10), transferred to a PVDF membrane and incubated with patient sera (1:200) and goat anti-human IgG-HRP (1:2000). Membranes were then visualised using a chemiluminescence system. For each age group, three example serum samples that returned a low OD against GST-PGP3 SW2 in the ELISA (Table 6.5A-C and Figure 6.5 A-C, B panels) and six example serum samples that returned a high OD above the 0.652 cut-off (Table 6.5A-C and Figure 6.5A-C, E panels) were assayed. In addition, three serum samples that returned an OD_{450} value just below the cut-off (Table 6.5 and Figure 6.5, C panels) and three serum samples that returned an

OD_{450} value just above the cut-off (Table 6.5 and Figure 6.5, D panels) were assayed. These example serum samples were selected based on the proximity of their OD to the 0.652 cut-off (Table 6.5).

For all age groups (Figure 6.5A-C), none of the serum samples that returned a low OD against GST-PGP3 SW2 in the ELISA reacted with the GST-PGP3 SW2 or GST antigens in the Western blots (Figure 6.5A-C, B panels). Many serum samples returned a high background in the Western blot. Across all age groups, of the total 9 example serum samples that returned an OD value just below the 0.652 cut-off in the ELISA, 7 did not react to GST-PGP3 SW2 in the Western blot (Figure 6.5, C panels). Sera nos. 1833 and 395 reacted weakly to the trimeric confirmation of GST-PGP3 SW2 (Figure 6.5A and C, C panels). All example serum samples across all age groups that returned an OD value just above the 0.652 cut-off in the ELISA reacted with GST-PGP3 SW2 in the Western blot (Figure 6.5A-C, D panels). Sera nos. 1836, 2075, 1613, 1539, 1495, 1353 and 574 reacted with trimeric GST-PGP3 SW2. Furthermore, sera nos. 1836, 1613, 1539, 1495, 326, 574 and 457 reacted to the monomeric confirmation of GST-PGP3 SW2. All example serum samples across all age groups that returned a high OD value in the ELISA reacted with GST-PGP3 SW2 in the Western blot (Figure 6.5A-C, E panels). Out of 18 serum samples assayed against GST-PGP3 SW2 in this group, 17 reacted with the trimer. Serum no. 1939 reacted with only the monomer of GST-PGP3 SW2 (Figure 6.5B, panel E). Additionally, serum no. 1704, 1196, 1456, 2064, 1943, 1939 and 397 reacted with the monomer of GST-PGP3. Although these serum samples reacted more profoundly to GST-PGP3 in the Western blot, sera nos. 1613, 1939 and 411 reacted weakly to the GST negative control antigen (Figure 6.5A-C). These samples returned background-corrected OD values of -0.009, 0.044 and 0.197, respectively, against GST in the indirect ELISA.

Table 6.5. Details of example sera from 13-15-year-olds assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST in a Western blot.

Example sera were assayed in the GST-PGP3 SW2 and GST ELISAs and were selected according to their background-corrected OD_{450} values and proximity to the OD cut-off. OD values shown are background-corrected values against GST-PGP3 SW2. 0.652 = cut-off for serum samples for females. 0.665 = cut-off for serum samples for males. GUM = genitourinary medicine; N = Non-GUM; NK = not known. The full details about each serum sample can be seen in appendix II. **[A]** 13-year-olds; **[B]** 14-year-olds; **[C]** 15-year-olds.

A

Figure 6.5A Panel	PHE ID / INC ID	Sex	Serum source	OD_{450}	OD Group
b	M200577 / 2177	M	NK	0.129	Low OD (negative)
	M187617 / 2118	M	NK	0.037	
	M201153 / 2108	M	N	0.021	
c	M201097 / 2106	F	NK	0.442	Just under cut-off (negative)
	M202963 / 2012	F	N	0.591	
	M193182 / 1833	F	N	0.568	
d	M193185 / 1836	F	N	0.662	Just above cut-off (positive)
	M190292 / 2075	M	NK	0.769	
	M182712 / 1613	F	NK	0.735	
e	M202965 / 2014	M	N	2.209	High OD (positive)
	M201387 / 1896	M	GUM	2.610	
	M194288 / 1887	M	N	1.827	
	M187382 / 1704	M	NK	2.067	
	M166764 / 1196	F	NK	3.008	
	M174541 / 1456	F	NK	2.534	

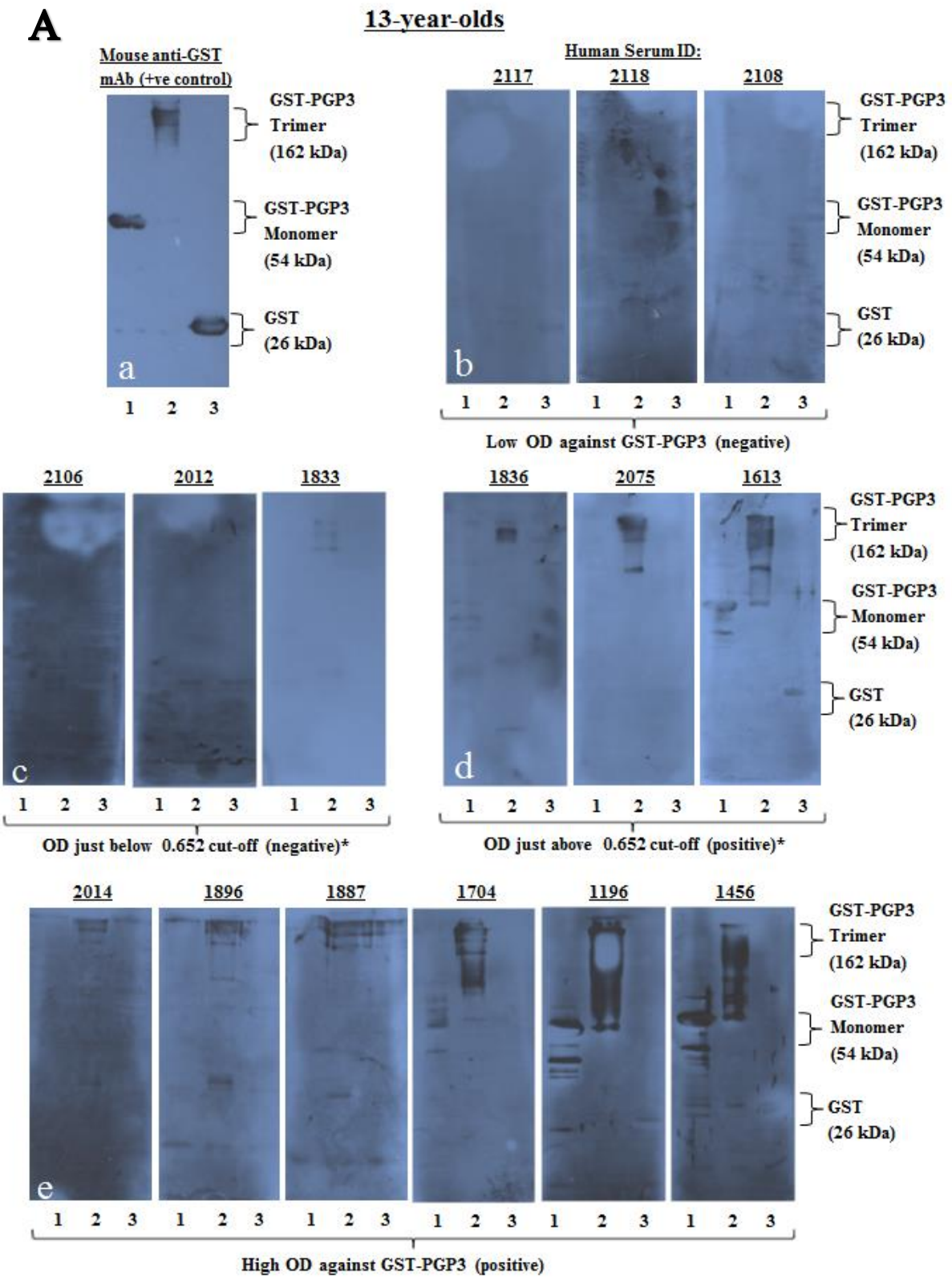
B

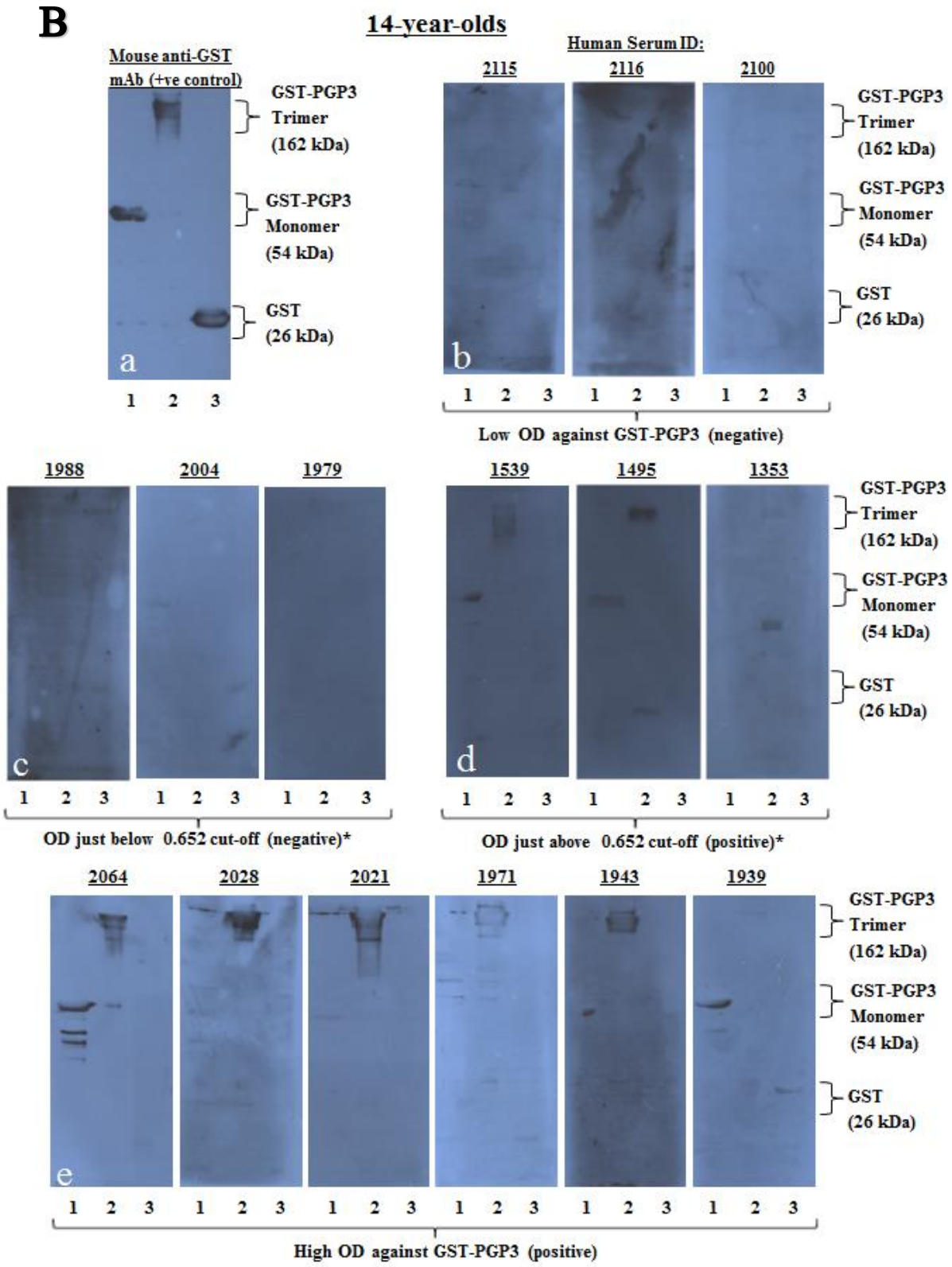
Figure 6.5A Panel	PHE ID / INC ID	Sex	Serum source	OD_{450}	OD Group
b	M200030 / 2115	F	N	0.033	Low OD (negative)
	M200031 / 2116	M	N	-0.077	
	M200953 / 2100	M	NK	0.024	
c	M201847 / 1988	F	N	0.539	Just under cut-off (negative)
	M202388 / 2004	M	GUM	0.498	
	M197375 / 1979	F	NK	0.454	
d	M179845 / 1539	M	NK	0.748	Just above cut-off (positive)
	M178035 / 1495	F	NK	0.964	
	M171673 / 1353	F	GUM	0.886	
e	M188542 / 2064	F	NK	3.325	High OD (positive)
	M189355 / 2028	F	GUM	2.513	
	M200030 / 2115	F	N	0.033	
	M200031 / 2116	M	N	-0.077	
	M200953 / 2100	M	NK	0.024	
	M201847 / 1988	F	N	0.539	

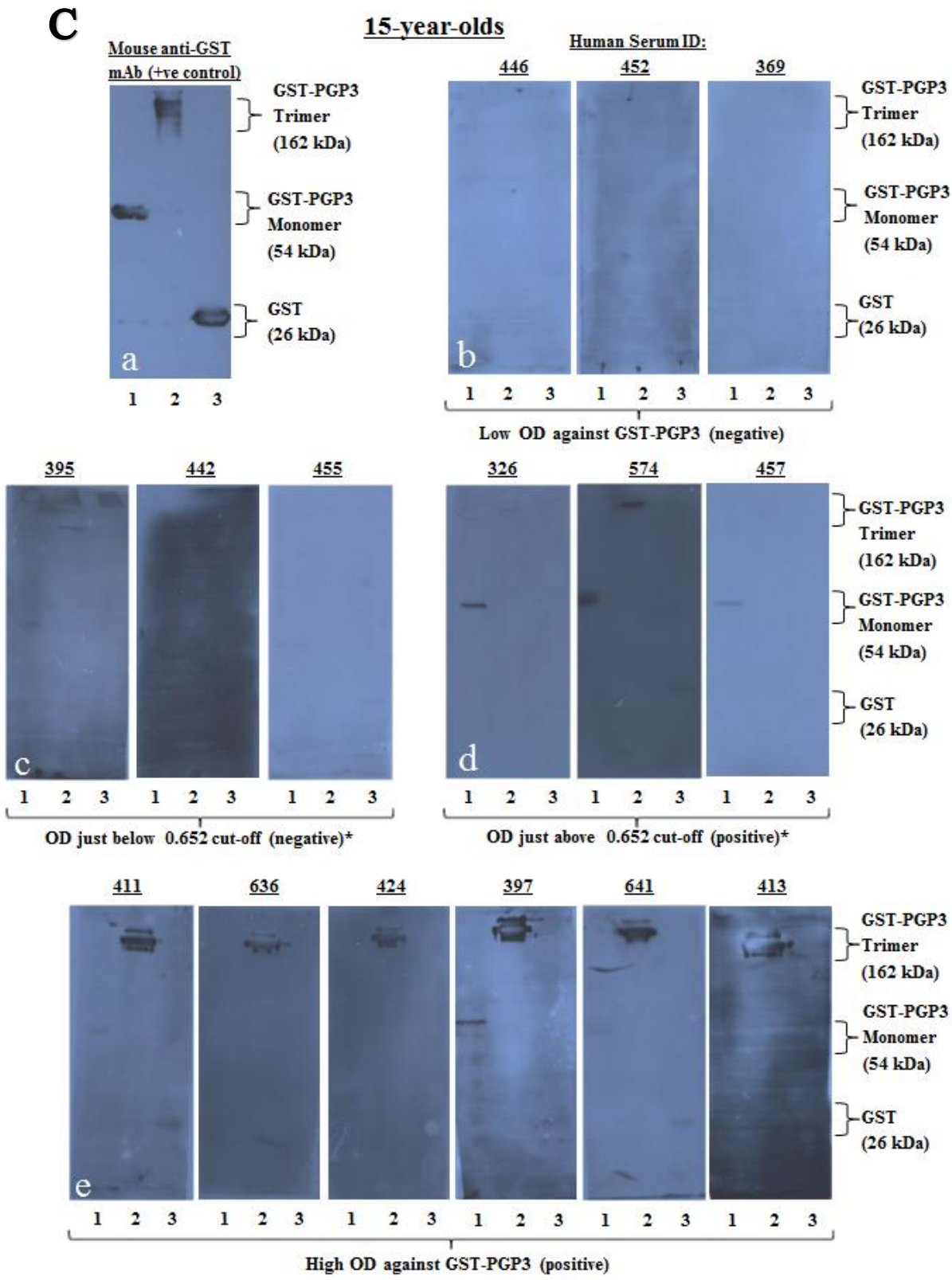
C

Figure 6.5A Panel	PHE ID / INC ID	Sex	Serum source	OD_{450}	OD Group
b	M178047 / 446	M	NK	0.146	Low OD (negative)
	M178477 / 452	F	NK	0.068	
	M173904 / 369	M	NK	0.080	
c	M174243 / 395	M	NK	0.616	Just under cut-off (negative)
	M176588 / 442	M	NK	0.645	
	M178532 / 455	F	NK	0.569	
d	M171253 / 326	M	NK	0.683	Just above cut-off (positive)
	M196215 / 574	M	N	0.780	
	M178716 / 457	M	NK	0.711	
e	M175561 / 411	F	GUM	3.33	High OD (positive)
	M189337 / 636	F	GUM	3.33	
	M175576 / 424	F	GUM	1.328	
	M174560 / 397	M	NK	2.237	
	M189621 / 641	F	N	2.533	
	M175563 / 413	F	GUM	2.948	

Figure 6.5. Examples of human antibody recognition of monomeric and trimeric PGP3 in a Western blot using sera from 13-15-year-old patients. 1 μg of recombinant GST-PGP3 from *C. trachomatis* pSW2 (serovar E) was denatured in 2% SDS and 1.25% BME and boiled for 5 minutes (lane 1) or left untreated (lane 2). GST was also included as a negative control (lane 3) and boiled with SDS and BME. A mouse monoclonal antibody to GST (mAb anti-GST) was used as a positive control to detect GST and monomeric and trimeric GST-PGP3 (panel a). Sera were assayed at 1:200. Sera were selected based on their background-corrected OD_{450} value when assayed against GST-PGP3 SW2 in our ELISA (Table 6.1). Three sera with a low OD and three sera with an OD just below the 0.652 cut-off were selected as negative controls (panels b and c, respectively). Three sera with an OD just above the 0.652 cut-off and three sera with a high OD were selected as positive controls (panels d and e, respectively). **[A]** 13-year-olds; **[B]** 14-year-olds; **[C]** 15-year-olds


A
13-year-olds
Mouse anti-GST mAb (+ve control)
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
GST (26 kDa)
a
1 2 3
Human Serum ID:
2117
2118
2108
b
1 2 3 1 2 3 1 2 3
Low OD against GST-PGP3 (negative)
2106
2012
1833
c
1 2 3 1 2 3 1 2 3
OD just below 0.652 cut-off (negative)*
1836
2075
1613
d
1 2 3 1 2 3 1 2 3
OD just above 0.652 cut-off (positive)*
2014
1896
1887
1704
1196
1456
e
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
High OD against GST-PGP3 (positive)


B
14-year-olds
Mouse anti-GST mAb (+ve control)
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
GST (26 kDa)
a
1 2 3
Human Serum ID:
2115
2116
2100
b
1 2 3 1 2 3 1 2 3
Low OD against GST-PGP3 (negative)
1988
2004
1979
c
1 2 3 1 2 3 1 2 3
OD just below 0.652 cut-off (negative)*
1539
1495
1353
d
1 2 3 1 2 3 1 2 3
OD just above 0.652 cut-off (positive)*
2064
2028
2021
1971
1943
1939
e
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
High OD against GST-PGP3 (positive)


C
15-year-olds
Mouse anti-GST mAb (+ve control)
Human Serum ID:
446
452
369
GST-PGP3 Trimer (162 kDa)
GST-PGP3 Monomer (54 kDa)
GST (26 kDa)
a
b
1 2 3
Low OD against GST-PGP3 (negative)
395
442
455
326
574
457
c
d
OD just below 0.652 cut-off (negative)*
OD just above 0.652 cut-off (positive)*
411
636
424
397
641
413
e
High OD against GST-PGP3 (positive)

6.3.4.1 Detection of anti-GST antibody positive in serum samples in an indirect ELISA and Western blot

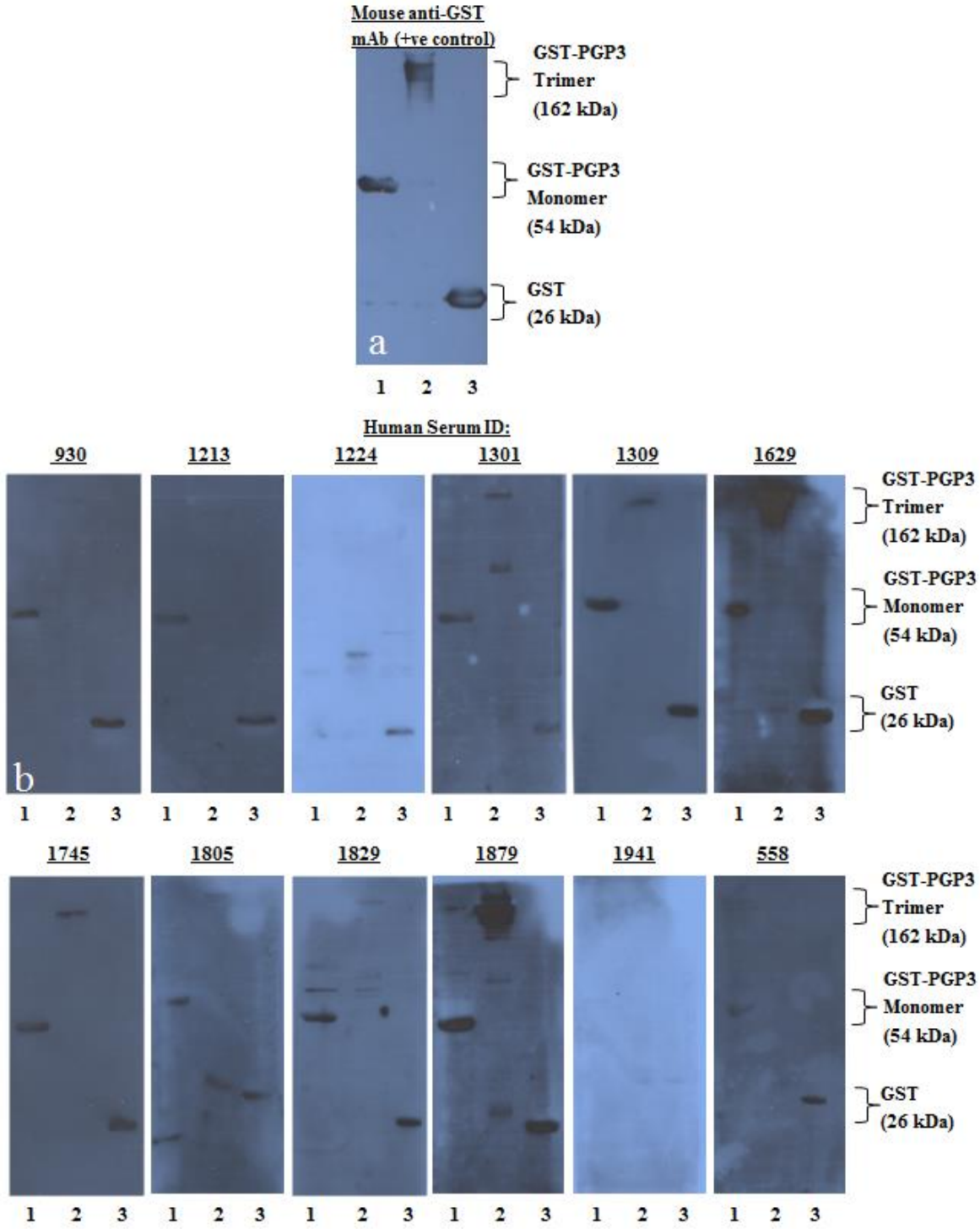
All serum samples that returned a background-corrected OD_{450} value above 0.450 were re-assayed in duplicate against GST-PGP3 SW2 in the ELISA. The sera were also assayed against the GST negative control antigen to assess potential cross-reactivity to the GST tag from the serum samples. To match the tag:polypeptide ratio of the GST-PGP3 antigen used in the ELISA and Western blots, the GST antigen was used at half the concentration of GST-PGP3 (5.2.3). In total, 395 samples were assayed against GST in the ELISA and 12 (3.04%; 0.57% of the total 2119 serum samples) returned high OD_{450} values (Table 6.6 and appendix VI). These samples were re-assayed for confirmation. These serum samples were then blotted against GST-PGP3 SW2 and GST in a Western blot, as described above (5.2.3). 11 samples reacted with the GST negative control antigen in the Western blot (Figure 6.6B). Only serum no. 1941 did not react to the GST or GST-PGP3 SW2 antigens. Although several serum samples returned a high background, reactions to both GST and GST-PGP3 antigens were observed. Serum no. 930, 1213, 1301, 1309, 1629, 1745, 1805, 1829, 1879 and 558 reacted to the monomer of GST-PGP3 SW2 and sera nos. 1301, 1309, 1629, 1745, 1805, 1829 and 1879 reacted to the trimer of GST-PGP3 SW2. These samples were considered positive for anti-GST antibodies and were not included in any of the above statistical analyses (6.3.1-6.3.3) (final serum total = 2107).

Table 6.6. Details of sera from 13-15-year-olds assayed against recombinant GST in a western blot.

Sera were assayed in the GST ELISAs and were selected for western blotting according to their background-corrected OD_{450} values. OD values shown are background-corrected values against GST. N = Non-GUM; NK = not known.

PHE ID / INC ID	Age	Sex	Serum Source	OD_{450}
M156429 / 930	14	F	NK	2.638
M167525 / 1213	13	M	NK	2.055
M167830 / 1224	13	M	NK	1.504
M170583 / 1301	13	M	NK	1.216
M170793 / 1309	14	M	NK	1.109
M183105 / 1629	13	M	NK	1.724
M198199 / 1745	14	F	N	2.652
M192013 / 1805	14	M	NK	1.632
M192961 / 1829	14	F	NK	1.424
M194278 / 1879	13	M	N	2.169
M196042 / 1941	14	M	N	1.272
M201561 / 555	15	M	N	0.753

Figure 6.6. Human antibody recognition of glutathione s-transferase in a western blot using sera from 13-15-year-olds. 1 μg of recombinant GST-PGP3 from *C. trachomatis* pSW2 (serovar E) was denatured in 2% SDS and 1.25% BME and boiled (lane 1) or left untreated (lane 2). 0.5 μg of recombinant GST was included (lane 3) and boiled with SDS and BME. A mouse monoclonal antibody to GST (mAb anti-GST) was used as a positive control to detect GST and monomeric and trimeric GST-PGP3 (panel a). Sera were assayed at 1:200. Sera that reacted to the GST negative control in the GST ELISA were assayed in a Western blot for confirmation (panel b).


Mouse anti-GST
mAb (+ve control)
GST-PGP3
Trimer
(162 kDa)
GST-PGP3
Monomer
(54 kDa)
GST
(26 kDa)
a
1 2 3
Human Serum ID:
930
1213
1224
1301
1309
1629
GST-PGP3
Trimer
(162 kDa)
GST-PGP3
Monomer
(54 kDa)
GST
(26 kDa)
b
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3
1745
1805
1829
1879
1941
558
GST-PGP3
Trimer
(162 kDa)
GST-PGP3
Monomer
(54 kDa)
GST
(26 kDa)
1 2 3 1 2 3 1 2 3 1 2 3 1 2 3 1 2 3

6.4 DISCUSSION

This is the first study to investigate the seroprevalence of anti-chlamydia antibodies in 13-15-year-olds in England. Little research has been undertaken to understand the seroprevalence of *C. trachomatis* in the young adult population. These samples were assayed against GST-PGP3 derived from *C. trachomatis* pSW2 (serovar E) in the indirect ELISA and validated using in-house controls. Antibody responses to GST-PGP3 were also confirmed by Western blot using example sera.

6.4.1 Determination of the seroprevalence of anti-PGP3 antibodies in 13-15-year-olds

The seroprevalence of antibodies to PGP3 in serum samples collected from male and female 13-, 14- and 15-year-olds not sourced from GUM clinics was 4.66%, 4.37% and 10.78%, respectively. The overall seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from an unknown or unrecorded source was 7.10%, 6.26% and 6.75%, respectively. Finally, the seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from GUM clinics was 18.52%, 18.18% and 24.32%, respectively. Overall seroprevalence in 13-, 14- and 15-year-olds from all combined serum sources was 6.87%, 6.70% and 10.47%, respectively.

The study was initially conducted to determine the seroprevalence of antibodies to PGP3 in serum samples collected from 15-year-olds in England. As the antibody prevalence was determined to be comparable to the 6-18% antibody seroprevalence rate in 17-18-year-olds reported by Horner *et al.* (165), the SEU approved our request to include serum samples from 13- and 14-year-olds in the study for further screening. This age group covers the critical time of earliest voluntary sexual debut when individuals are likely to be exposed to chlamydia for the first time (176). In 2009, Wills *et al.* (118) assayed 747 sera from children aged 2-13-years-old as presumed negative controls for their ELISA and 25 (3.34%) were found to be positive for *C. trachomatis* antibodies. It is possible for children to acquire chlamydial antibodies through vertical transmission of *C. trachomatis* infection (54, 55), maternal antibody transmission (189, 190) or sexual abuse (178, 191). Furthermore, in 2013 Horner *et al.* (164) reported that PGP3 antibody decreases over time and these antibodies were still detectable even after 10 years since the last diagnosed urogenital infection in adult patients. As the GST-PGP3 SW2 ELISA was an overall 10.28% more sensitive than the ELISA described by

Wills *et al*., we therefore hypothesised that the seroprevalence rate of 13- and 14-year-olds would be higher than the 3.34% rate reported by Wills *et al*., but lower than the seroprevalence rates found in 15-year-olds in our study. The seroprevalence of antibodies in samples collected from 13- and 14-year-olds not sourced from GUM clinics, 4.66% and 4.37%, was similar to the percentage of sera from 2-13-year-olds used as negative controls in the Wills *et al.* ELISA found to be positive for *C. trachomatis* antibodies, a difference of 1.32% and 1.03%, respectively. These could potentially act as baseline percentage rates for the under 16s population to include individuals who have gained antibodies through vertical or maternal transmission or through sexual abuse. Hence, the difference between the 'baseline' seroprevalence rate and the determined seroprevalence of 10.78% in samples collected from 15-year-olds not sourced from GUM clinics could be the more accurate percentage of individuals who have acquired antibodies through unprotected sexual debut or further sexual abuse in this age group. Furthermore, as the overall sensitivity of our GST-PGP3 SW2 ELISA was 68.18% in comparison to diagnosis by NAATs, the seroprevalence rates of anti-PGP3 antibodies within serum groups may be higher than determined. In addition, as previously discussed, some patients with a chlamydial infection may not have seroconverted at the time of serum collection. An important consideration is that due to the higher sensitivity for female serum samples in the GST-PGP3 SW2 ELISA in comparison to male samples (71.93% vs 64.15%), higher determined seroprevalence rates in females in comparison to males may not necessarily represent a higher prevalence of prior exposure to chlamydia within the female population.

As all details of the sera were blinded until all assays were completed, the sources of collection of the sera were not known. We initially received 708 serum samples collected from 15-year-olds from the SEU. Unbeknownst to us, 111 samples from this age group were sourced from GUM clinics. The overall prevalence of anti-PGP3 antibodies in these samples sourced from this serum group was 24.32%. However, as the samples were blinded during screening, these samples acted as a control group as samples sourced from the GUM clinic would have an increased likelihood of containing anti-PGP3 antibodies due to presumed sexual activity or sexual abuse. A limitation to this study was the number of serum samples from an unknown source. A total of 1379 serum samples were from an unknown or unrecorded source, whilst 547 samples were known to be from a non-GUM clinic source. Some of the samples from an unknown

source may have been sourced from a GUM clinic. Nonetheless, the overall seroprevalence rate of anti-PGP3 antibodies in serum samples collected from 15-year-olds that were not sourced from a GUM clinic was higher than expected at 10.78% (18/167). However, only 167 samples were sourced from this serum group. An advantage of the Chi-square for association/trend and Fisher's exact statistical tests are that raw numbers of observations (not percentage prevalence) are used for calculating potential statistical significances between serum groups and therefore sample sizes are taken into consideration. For example, comparison of antibody prevalence rates of 2/80 (2.50%) and 7/67 (10.45%) between 14- and 15-year-old females, respectively, sourced from non-GUM clinics did not reveal a statistical significance ($p = 0.080$). Although the prevalence rates between both groups are observable, the most likely possibility for no statistical significance was the small sample size and number of positive samples found in the serum groups. By contrast, comparison of antibody prevalence rates of 3/103 (2.91%) and 11/100 (11.00%) between 13- and 15-year-old males, respectively, from a higher number of serum samples sourced from non-GUM clinics revealed statistical significance ($p = 0.027$).

Small sample sizes and number of positive samples may also be an explanation for no statistical significances found between serum groups in tests for associations between antibody prevalence and year of serum sample collection (p values = 0.335-0.539). Horner *et al.* (2013) assayed over 4,732 serum samples for anti-PGP3 antibodies from females aged 17-24-years-old and found some association between antibody prevalence and year of sample collection. However, sample sizes between each serum group and the number of positive samples found were higher than those used in our study. Small sample sizes and number of positive samples may be also an explanation for no statistical significances found when comparing serum groups to test for associations between antibody prevalence and location of serum sample collection (p values = 0.062-0.847). As our aim was to determine the seroprevalence rate of anti-PGP3 antibodies within 13-15-year-olds in England, we required serum samples to be sourced from across various regions of England. Samples provided by the SEU had been sourced from various counties, cities and hospitals across England. Using samples already stored at the SEU eliminated the need to recruit patients, as collecting 2119 samples would have been very time consuming and potentially costly. However, a disadvantage of using these samples was the restriction of the source of the serum collection and the

region. Of the 2107 serum samples received from the SEU and used in the analysis, a total of 770 were sourced from Manchester. By contrast, only 12 samples were sourced from Poole, Dorset. Serum samples sourced from Birmingham were found to have the highest seroprevalence rate of 9.23%. As it is known that the detection rate of chlamydia is higher in larger cities than in rural regions of England (89), this may account for the higher than expected seroprevalence rate of anti-PGP3 antibodies seen in serum samples collected from larger cities.

6.4.2 Validation of assay results using in-house controls and Westgard rules

As described in chapter five (5.4.3), there are no 'gold-standard' guidelines to calculate the reproducibility of a serological assay. Likewise, there are no standard guidelines to validate the results of an assay run. The Westgard multirule QC system is a set of rules used to judge the validity of a set of results from an assay run. As the Westgard rules are only based on a recommended 2-4 controls, and as there are no other official guidelines or recommendations on the number or types of controls to use, we selected our controls based on recommendations from Dr. Ezra Linley, Public Health England (email communication). Dr Linley recommended that we use three positive control serum samples that return low, medium and high OD_{450} values against GST-PGP3 SW2 in the indirect ELISA. However, we decided to not only use Dr. Linley's recommendations but also include a combination of various controls that included three positive control sera, three negative control sera and two positive monoclonal and polyclonal control antibodies that produce a range of OD_{450} values against GST-PGP3 in the ELISA. The aim of using a higher number of a combination of controls was to give a detailed analysis of each assay run conducted. For each assay run, new reagents were prepared and manual plate washing and pipetting was conducted so it was considered necessary to validate each validate using well-characterised controls. No other chlamydial seroprevalence study has described how their results were validated.

6.4.3 Human antibody recognition of PGP3 is not dependent the trimeric structure of PGP3: further evidence

Example sets of sera were assayed in a Western blot against monomeric GST-PGP3, trimeric GST-PGP3 and the GST negative control antigen to assess their reactivity to the recombinant GST-PGP3 antigen. As the Western blot procedure is time-consuming and laborious, only a small subset of samples from each age group were assayed. Three

sera that returned a low OD, three sera that returned OD values just below the cut-off, three sera that returned OD values just above the cut-off, and six sera that returned high ODs against GST-PGP3 SW2 were assayed. All sera above the cut-off reacted with GST-PGP3. Furthermore, of the 29 sera that reacted to GST-PGP3 by Western blot, 14 serum samples reacted to the monomer of GST-PGP3. This further contradicts the findings by Chen *et al.* (2010) and Li *et al.* (2008) (125, 153) as their findings suggest that human antibody recognition of PGP3 is dependent on the trimeric structure of PGP3. Two serum samples that returned an OD value just under the cut-off reacted weakly with the trimer of GST-PGP3 by Western blot. As the overall sensitivity of the GST-PGP3 SW2 ELISA was 68.18%, the actual seroprevalence rate of anti-PGP3 antibodies within our serum cohort may be higher. This may explain why some sera returned a positive result by Western blot but a negative result in the GST-PGP3 SW2 ELISA.

6.4.4 The inclusion of GST as a negative control excludes false-positive reactivity

Using recombinant GST-tagged PGP3 as the detection antigen in the indirect ELISA assay allowed the inclusion of recombinant GST as a negative control antigen. Although no serum samples used in the ELISA optimisation or validation studies or any Western blotting experiments reacted with the GST negative control antigen, we still considered it necessary to assay all sera collected from 13-15-year-olds against GST that reacted against recombinant GST-PGP3 SW2 in our ELISA. This was to exclude the possibility of potential false-positive results due to cross-reactivity with the GST tag from the serum samples; a negative reaction to GST and a positive result for GST-PGP3 would be hence be due to the presence of anti-PGP3 antibodies in the serum sample. The importance of GST as a negative control was confirmed when 12 serum samples returned a high background-corrected OD value against GST in the ELISA, of which 11 samples reacted to GST by Western blot. As recombinant GST expressed using pGEX expression vectors (GE Healthcare) is derived from *Schistosoma japonicum*, the presence of antibodies to GST is very unlikely, but not impossible, as this causative agent of schistosomiasis is found in rural regions of Asia, particularly China and the Philippines (185, 192).

An indirect ELISA to detect antibodies to PGP3 was validated using serum samples from patients diagnosed positive or negative for *C. trachomatis* by NAAT and a cut-off

OD value was established. As the GST ELISA was not validated using characterised sera, a limitation of the GST ELISA is therefore the lack of a cut-off OD value. However, excluding the 12 sera that were assayed by Western blot, all other sera assayed against GST returned background-corrected OD values under 0.527 in the GST ELISA. The use of GST as a fusion protein to detect antibodies to PGP3 has previously been described (159, 167). Furthermore, the use of GST as a fusion protein to detect antibodies in human sera to other antigens has been described, such as human papillomavirus type 16 (193, 194) and dengue viral proteins (195). GST as a negative control in PGP3 studies has been described (125, 153, 159). However, it has not been described as a negative control in chlamydial seroprevalence studies. It has also been applied as a negative control in a GST fusion multiplex assay (196), although the method of background correction involved subtracting the Mean Fluorescent Intensity (MFI) of the GST tag from the raw MFI. This method of background correction has also been described in other ELISAs (197, 198). The OD value against GST was not used for background correction in the GST-PGP3 ELISA; the OD value of serum non-antigen-coated wells was subtracted from serum antigen-coated wells, as described by Bas *et al.* (2001) in their PGP3 ELISA (122). This may explain why serum no.1941 returned a background-corrected OD value of 1.272 in the GST ELISA but did not react to GST in the Western blot. However, other serum samples returned a similar OD value against GST in the ELISA reacted to GST by Western blot. Using the OD value of GST to correct for background would not allow for the detection of false-positives caused by cross-reactivity to the GST tag. Saijo *et al.* (197) expressed carboxy-terminal halves of nucleoproteins of Ebola virus and Marburg virus as GST fusions as detection antigens in a Western blot and GST was included as a negative control. Of the 26 patient serum samples assayed, 1 sample reacted to GST by Western blot and this was excluded from any further experiments, although no further discussion or explanation for the possible cross-reactivity was given. The GST expressed in the study described by Saijo *et al.* was expressed from the same pGEX family of expression vectors used in our study. Matsunaga *et al.* (199) also described the cross-reactivity of sera to the GST tag as a negative control in a Western blot. However, the data was not shown nor discussed. Furthermore, Matsunaga *et al.* also considered reactions to be positive to the antigen of interest in a Western blot if sera returned weak reactions to GST but strong reactions to the antigen of interest. We also used this method when three serum samples returned weak reactions to GST in our Western blot but strong reactions to GST-PGP3; these

were considered positive for anti-PGP3 antibodies. Likewise, serum samples that reacted strongly to GST by Western blot may nonetheless also contain antibodies to PGP3. In summary, the experiments described in this chapter were not the first to identify human sera cross-reactive to the GST fusion tag. However, due to the method of background correction using GST in the studies described above, further serum samples containing antibodies to GST may have gone undetected in the studies.

6.4.5 Conclusions

- For the first time, the seroprevalence rate of anti-PGP3 antibodies within serum samples collected from 13-15-year-old children in England has been determined using a properly designed systematic survey:
    - This is the first chlamydial seroprevalence study incorporating PGP3 ELISAs to describe the inclusion of a well-characterised set of controls and a multirule quality control method system to validate the results of day-to-day assay runs.
    - We have provided further evidence that human antibody recognition of PGP3 is not dependent on the trimeric structure of PGP3.
    - The inclusion of GST as a negative control in the indirect ELISA is an essential control to assess potential false-positive reactivity to the GST tag.
    - Seroprevalence of antibodies in 13-, 14- and 15-year-olds in samples not sourced from a GUM clinic was 4.66%, 4.37% and 10.78%, respectively.
    - Seroprevalence of antibodies in 13-, 14- and 15-year-olds in samples from an unknown/unrecorded source was 7.10%, 6.26% and 6.75%, respectively.
    - Seroprevalence of antibodies in 13-, 14- and 15-year-olds in samples sourced from a GUM clinic was 18.52%, 18.18% and 24.32%, respectively.
    - Overall seroprevalence in 13-, 14- and 15-year-olds from all combined serum sources was 6.87%, 6.70% and 10.47%, respectively.
    - The seroprevalence of antibodies to PGP3 in 15-year-olds found in this study support the antibody prevalence rates of 6-18% found in 17-18-year-olds (165) reported by Horner *et al.*(2013).

CHAPTER SEVEN: FINAL DISCUSSION

Urogenital infection with *Chlamydia trachomatis* is the most commonly diagnosed sexually transmitted infection in the developed world. 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 (48). The treatment and management of these preventable chlamydial infections and their associated comorbidities place a significant financial burden on healthcare systems. The National Chlamydia Screening Programme was established in England in 2002 to provide opportunistic chlamydial screening to sexually active patients aged 15-24-years-old. However, the justification for the cut-off at age 15 is unclear, despite evidence of increasingly earlier and unprotected sexual debut (98, 176-178). Furthermore, patients aged 15 and under are often only tested under special circumstances and parental notification is often encouraged in accordance with Fraser Guidelines (109). As the NSCP relies on opportunistic testing and many cases of chlamydia are asymptomatic, diagnoses made by nucleic acid amplification testing in the under 16s are very likely an underrepresentation of actual chlamydial prevalence. Understanding the seroprevalence of urogenital *C. trachomatis* infection in this population is essential as this would support targeted intervention and enhanced screening. This thesis describes the development and validation of a sensitive and specific ELISA to detect anti-PGP3 antibodies in patient sera and the application of this ELISA to detect antibodies in sera collected from 13-15-year-olds from England to determine the seroprevalence of *C. trachomatis* within this population. Until now, no systematic investigation of the seroprevalence of *C. trachomatis* in the under 16s population in England had been conducted.

7.1 PGP3 ELISAS: DEVELOPMENT, VALIDATION AND FURTHER STUDIES

A serological assay to measure the presence of antibodies to *C. trachomatis* as an indicator of current or prior infection was the most appropriate method to determine the prevalence of *C. trachomatis* in the under 16s as a majority of patients infected with chlamydia produce antibodies to chlamydial antigens (75, 118, 123, 160, 163, 167, 168). Serological assays incorporating recombinant PGP3 have so far proven to be an effective tool to detect prior *C. trachomatis* exposure (164, 165, 169-172). However, the ELISAs developed lacked standardisation and there was significant variation between the assay methods, including origin of the PGP3 and recombinant protein purification

methods. We developed a simple, reproducible protocol to express and purify recombinant PGP3 to incorporate into the ELISA (chapter three). As the PGP3 ELISA developed by Wills *et al.* (118) was based on recombinant PGP3 derived from the less common *C. trachomatis* L1 serovar (pLGV440), we expressed and purified PGP3 derived from the L1 serovar and the more common serovar E (pSW2) to compare assay sensitivity when used as capture antigens to detect anti-PGP3 antibodies in patient sera (chapter five). Both recombinant proteins were initially purified with a retained 7 kDa N-terminal polyhistidine tag. However, analysis by Western blot (figure 3.15) showed that recombinant PGP3 derived from *C. trachomatis* serovar L1 was not able to trimerise whereas PGP3 derived from serovar E was able to form a trimer. Furthermore, during initial ELISA development (chapter four), ELISA results also revealed that sera collected from patients diagnosed positive for *C. trachomatis* by NAAT reacted with his-PGP3 derived from serovar E but not his-PGP3 derived from L1.

The decision was then made to express and purify PGP3 derived from serovar E and L1 with an N-terminal GST tag. Although purification of recombinant GST-PGP3 was successful using affinity chromatography, the process took approximately one working week, was labour intensive and a 250 ml bacterial culture resulted in typical yields of 600 µg of protein. Ideally, for future experiments and less labour this process should be scaled up to produce higher yields of recombinant protein and the protein should be purified further using high performance liquid chromatography (HPLC) such as size-exclusion chromatography. Despite the larger N-terminal 26 kDa fusion tag of the GST-PGP3 proteins, both recombinant proteins were able to trimerise and bind anti-PGP3 antibodies in an ELISA and Western blot using sera collected from patients diagnosed positive for *C. trachomatis* by NAAT. However, validation of both ELISAs incorporating GST-PGP3 derived from serovar E and L1 using a larger cohort of sera revealed no statistically significant differences in sensitivities (chapter five). Despite this, overall sensitivity for the GST-PGP3 SW2 ELISA was marginally higher at 68.18%, a difference of 1.8%. Furthermore, as serovar E is the most common urogenital tract serovar in the developed world (31, 32), we made the decision to continue further ELISA and Western blot experiments with recombinant PGP3 derived from serovar E. PGP3 derived from serovar E (pSW2; EMBL: 865439) shares a 100% amino acid identity with PGP3 derived from the next most common serovars D (pCHL1; Genbank: NC_001372) and F (pSW4; EMBL: 865441). Bas *et al.* (122), Ghaem-Maghami *et al.*

(163), Donati *et al.* (160), Goodhew *et al.* (167) and Comanducci *et al.* (75) had previously developed assays incorporating recombinant PGP3 derived from *C. trachomatis* serovar D, whereas Wills *et al.* (118) had incorporated PGP3 from serovar L1. Horner *et al.* (172) has recently developed a double-antigen ELISA incorporating recombinant PGP3 which reported a specificity of 97.8% and sensitivities of 82.9% and 54.4% for female and male samples, respectively. However, although the derivation of the serovar was not stated, the Wills *et al.* ELISA was referenced in the methods and therefore it can be perhaps be assumed that the PGP3 is again derived from the L1 serovar. Wills *et al.* and Horner *et al.* had used a large cohort of sera of over 1100 and 800 samples (Table 1.5), respectively, to validate their assays. If we had used a similar number of serum samples to validate our GST-PGP3 SW2 and GST-PGP3 LGV440 indirect ELISAs, a statistically significant difference in sensitivities between the assays may have been observed. However, this may have taken some time as it took several months to collect approximately 180 serum samples from patients diagnosed positive/negative for *C. trachomatis* by NAAT used for the ELISA validation studies in chapter five. Furthermore, the nine varying amino acids between PGP3 derived from *C. trachomatis* serovars E and L1 may be hidden within the PGP3 trimer (figure 1.7C) and may not be accessible to antibodies during chlamydial infection. Therefore, calculating differences in sensitivities between the GST-PGP3 SW2 and GST-PGP3 LGV440 indirect ELISAs may not be feasible.

As previously discussed, there is significant variation in the methods used to detect anti-PGP3 antibodies in patient serum samples using serological assays incorporating PGP3 as the capture antigen (75, 118, 122, 160, 163, 167, 172). These methods include the assay platform, blocking conditions, the method of background correction, the PGP3 *C. trachomatis* serovar derivation, method of antigen purification, numbers and sources of positive and negative controls and methods of control serum characterisation. The method of sourcing the sera to validate the ELISAs was of particular interest, as no official guidelines or an established bank of well-characterised serum samples currently exist for assay validation. This is most apparent in the validation methods of the PGP3 assays, as shown in detail in Table 1.4 (chapter 1); Goodhew *et al.* (167) only used 11 positive serum positive to validate their multiplex assay, whereas Wills *et al.* (118) used 356 positive serum samples for validation of their indirect PGP3 ELISA. We selected serum samples from presumably sexually active adult patients diagnosed positive for *C.*

trachomatis by NAAT to increase the likelihood of the serum samples containing antibodies to PGP3. Some difficulty was encountered when selecting negative control serum for ELISA validation as there was no guarantee of the absence of anti-PGP3 antibodies in serum samples collected from a patient of any age and from any clinical setting (GUM clinic/non-GUM clinic (figure 4.9)). Using serum samples collected from children would not guarantee the absence of anti-PGP3 antibodies (118) (167) as it is possible for children to acquire anti-PGP3 antibodies through vertical transmission of *C. trachomatis* infection (54, 55), maternal antibody transmission (189, 190) or sexual abuse (178, 191) and it is not known how long anti-PGP3 antibodies would remain detectable. Furthermore, since we aimed to develop an ELISA to detect anti-PGP3 antibodies in serum samples collected from patients under 16 as part of a larger seroprevalence study, using serum samples collected from patients under 16 to validate the GST-PGP3 SW2 and GST-PGP3 LGV440 ELISAs would not have been an appropriate method; the main advantage of using serum samples from GUM patients was that chlamydial diagnosis could be confirmed or ruled out using NAAT. Some studies obtained negative serum cohorts based on *C. trachomatis* diagnosis by NAAT (118, 122, 163, 167, 172), presence of symptoms (122) or isolation of chlamydia by culture (160). Bas *et al.* (122) obtained their negative serum cohorts from 'healthy donors' but did not confirm the absence or presence of *C. trachomatis* using any other methods. We chose to select serum samples from adults diagnosed negative for *C. trachomatis* by NAAT to validate the ELISAs. However, 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 therefore these serum samples may have had a potential to contain anti-PGP3 antibodies from previous *C. trachomatis* exposure. However, using the patient NAAT result was the most appropriate option to characterise the serum samples as there was no other validated and suitable commercial assay available to confirm the absence of other chlamydial antigens in patient sera and hence confirm the absence of prior chlamydial exposure.

The method of serum characterisation may explain why the specificity of our cleaved PGP3 SW2 ELISA was reduced; to investigate the possibility that the N-terminal GST tag was lowering the sensitivity of the assay, the ELISA validation was repeated using

the same serum samples but instead using PGP3 derived from serovar E that had been subsequently cleaved from the GST tag using a Prescision Protease enzyme. This was to ensure that PGP3 alone would saturate the assay as it was discovered that increasing the antigen concentration resulted in higher OD_{450} values obtained from assaying sera from patients positive for *C. trachomatis* by NAAT (figure 4.6). However, the specificity of the assay was 87.50%, 10.50% lower than the specificity of the GST-PGP3 SW2 ELISA (figure 5.4). As the concentration of the PGP3 in the PGP3 SW2 ELISA was approximately twice that of the GST-PGP3 SW2 ELISA, the reduced specificity may have been due to the increased concentration of PGP3 detecting lower levels of anti-PGP3 antibodies in the serum samples collected from patients diagnosed positive for *C. trachomatis* by NAAT. This could be investigated further by first characterising the serum samples by confirming the absence of anti-chlamydial antibodies by MIF and then re-validating the PGP3 SW2 ELISA to investigate if the sensitivity of the ELISA has been increased. Although the seroprevalence studies using serum samples collected from 13-15-year-olds has been completed using the GST-PGP3 SW2 ELISA (chapter six), an ELISA that demonstrates a higher sensitivity and specificity would be helpful for future seroprevalence studies of other population groups.

In conclusion, the standardisation of methods, including antigen choice, assay platform, background correction and blocking method used to validate newly developed serological assays to detect antibodies in patient serum samples as an indicator of prior infection to *C. trachomatis* is something that needs to be addressed, with particular attention to the methods used for selecting patient serum samples for ELISA validation.

7.2 THE SEROPREVALENCE OF *C. TRACHOMATIS* IN 13-15-YEAR-OLDS AND FUTURE STUDIES

As many *C. trachomatis* cases are asymptomatic and 15-year-old patients are only screened for urogenital chlamydial infection under special circumstances (109), conducting a seroprevalence study was the most appropriate method to determine the prevalence of chlamydia within this population. The study conducted in chapter six was the first to investigate the seroprevalence of anti-chlamydia antibodies in 13-15-year-olds in England. Prior to this study, little research had been undertaken to understand the seroprevalence of *C. trachomatis* in the young adult population. The seroprevalence of antibodies to PGP3 in serum samples collected from male and female 13-, 14- and

15-year-olds not sourced from GUM clinics was 4.66%, 4.37% and 10.78%, respectively. The overall seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from an unknown or unrecorded source was 7.10%, 6.26% and 6.75%, respectively. Finally, the seroprevalence of antibodies in samples collected from 13-, 14- and 15-year-olds sourced from GUM clinics was 18.52%, 18.18% and 24.32%, respectively. Overall seroprevalence in 13-, 14- and 15-year-olds from all combined serum sources was 6.87%, 6.70% and 10.47%, respectively. Confirming our hypothesis (1.14.3), the antibody prevalence of these serum groups collected from only the 15-year-old cohort was determined to be comparable to the 6-18% antibody seroprevalence rate in 17-18-year-olds reported by Horner *et al.* (165). Although 15-year-olds are covered for screening by the NCSP, they are the only age group subject to the Fraser guidelines and our data suggests that the same standards of screening currently applied to the 16-24-year-old age group should also be applied to 15-year-olds.

The Fraser guidelines provide guidance to health professionals in relation to providing and advising contraceptives to the under 16s and, as of 2006, treating sexual health issues (109). The guidelines also state that the health professional can only provide treatment and screening if they are satisfied that the patient cannot be persuaded to inform their parents that he or she is seeking contraceptive advice and/or STI screening. Although there is little evidence in the literature to suggest that patients aged 15-years-old and under may be discouraged from attending chlamydial screening or seeking contraceptive advice from health professionals due to these guidelines, a survey to investigate the attitudes of this age group towards chlamydial screening may be helpful in determining if asking the patient for parental notification may discourage chlamydial screening in these age groups.

Despite evidence of increasing sexual debut in the 13-14-year-old age groups (176), ages not targeted by the NCSP, only 7,249 patients were screened at a GUM clinic for chlamydia in England in 2015, of which 6,094 were female (200). Of the total 7,249 patients screened, over 674 (9.30%) were diagnosed positive for urogenital chlamydial infection by NAAT. In chapter six, we found the antibody seroprevalence rates in 13- and 14-year-olds sourced from GUM clinics to be 18.52% and 18.18%, respectively. However, the seroprevalence of antibodies in 13- and 14-year-old patients not sourced from GUM clinics was 4.66% and 4.37%, respectively, at least 6.12% lower than the

rate found in 15-year-olds. This data suggests that chlamydial screening by the NCSP may not need to be extended to 13-14-year-olds but instead that screening uptake amongst those who have undergone sexual debut may need to be increased by means of improving sex and relationship education (SRE) in these age groups.

Improving SRE provided to 13-14-year-olds and 15-year-olds would complement increasing chlamydial screening amongst the patients and this could be done by providing education to the patients either through the schools, the parents or through healthcare settings (201). In 2016, the Terrence Higgins Trust reported that SRE is inadequate or absent in many schools (202). The "SRE: Shh…No Talking" report surveyed over 900 16-24-year-olds and 99% of respondents believed that SRE should be compulsory in all schools. By contrast, 1 in 7 respondents had not received any SRE throughout their schools years and over half of those who did rated their SRE as very poor. In 2011, Samkange-zeeb *et al.* published a systematic review to determine the level of STI knowledge of adolescents in Europe (203). In Sweden, Höglund *et al.* (204) found that only 46% of those surveyed were aware that chlamydia could be asymptomatic. In the UK, although Garside *et al.* (205) found that 97% of female year 9 pupils and 100% of female year 11 pupils had awareness of HIV, only 41.4% and 22.7%, respectively, had awareness of chlamydia. Although the UK has very recently introduced compulsory SRE to children aged four and above in all schools, including free schools and academies (206), parents still have the right to withdraw their child from SRE. Research has shown that parents may be hostile or critical towards teachers or the SRE curriculum if they do not deem it to be 'age-appropriate' material (207). However, the higher than expected seroprevalence rates found in 15-year-olds (chapter six) and the patient demand for compulsory and high quality SRE in all schools (202) may support the need for compulsory SRE in at least all 15-year-olds irrespective of parental consent or faith school status (208).

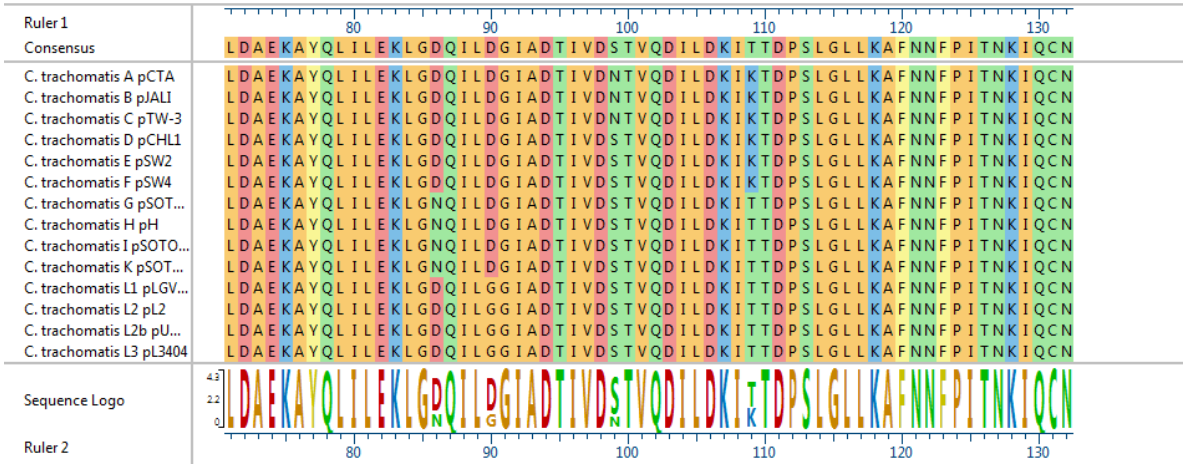
As previously discussed, it is possible for children to acquire chlamydial antibodies through vertical transmission of *C. trachomatis* infection (54, 55), maternal antibody transmission (189, 190) or sexual abuse (178, 191). Although the study was not intended as a seroprevalence study, in 2009 Wills *et al.* assayed 747 sera from children aged 2-13-years-old as presumed negative controls for their ELISA and 25 (3.34%) were found to be positive for *C. trachomatis* antibodies. The seroprevalence of antibodies in samples collected from 13- and 14-year-olds not sourced from GUM

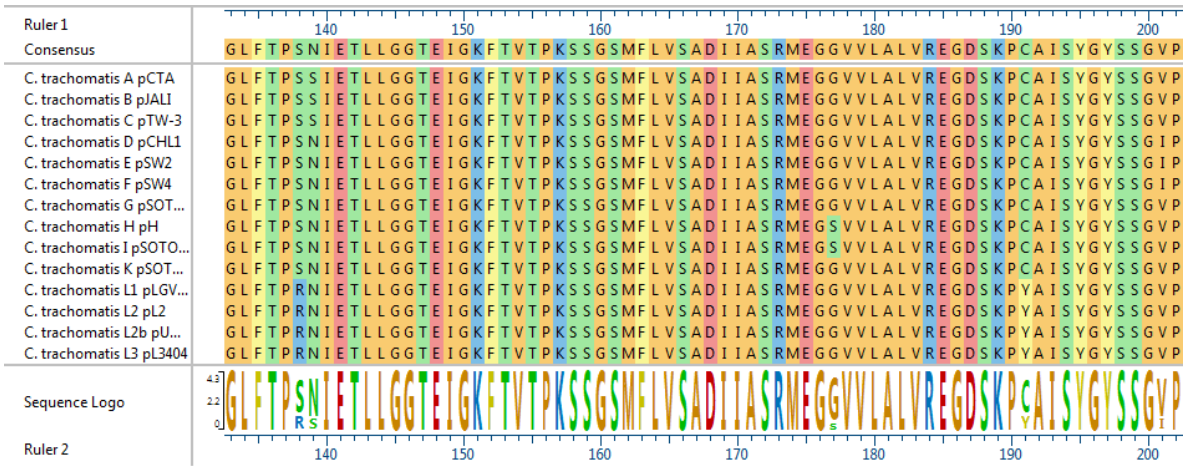
clinics, 4.66% and 4.37%, respectively, was similar to the percentage of sera from 2-13-year-olds used as negative controls in the Wills *et al.* ELISA found to be positive for *C. trachomatis* antibodies, a difference of 1.32% and 1.03%, respectively. As discussed, these could potentially act as baseline percentage rates for the under 16s population to include individuals who have gained antibodies through vertical or maternal transmission or through sexual abuse. However, as the Wills *et al.* study included an overall antibody percentage rate obtained from patients aged 2-13-years-old and it is unclear how the sera were distributed in each age group, the percentage rate from those samples could not be compared with the seroprevalence rates found in 13- and 14-year-olds in our study. Additionally, the Wills *et al.* PGP3 ELISA was an overall 10.28% less sensitive than the GST-PGP3 SW2 ELISA. Furthermore, as the Wills *et al.* ELISA only included samples for the purpose of ELISA validation and was not a seroprevalence study, a study consisting of sera purposefully collected from patients aged 0-12-years-old for determining the seroprevalence of *C. trachomatis* within this population would be a more accurate method of determining a baseline level of anti-PGP3 antibodies. However, obtaining the necessary ethical permission due to the ages of the patients may be difficult. Obtaining the numbers of serum samples required for statistical significance due to the ages of the patients may also be difficult as the seroprevalence rate of antibodies in younger age groups will likely be lower. Furthermore, in 2013 Horner *et al.* (164) reported that PGP3 antibody decreases over time and these antibodies were still detectable even after 10 years since the last diagnosed urogenital infection in adult patients. However, it cannot be certain that the patients in this study were not re-infected during the time period. Therefore, using the GST-PGP3 SW2 ELISA, it may be possible to measure the primary antibody response and predict the level of depletion of anti-PGP3 antibodies and also the rate of seroconversion following urogenital chlamydial infection using animal studies. A study using mice has already shown that it is possible for chlamydial antibodies to be passed onto newborns (189) but a study that would also monitor the level of antibody depletion in newborns and infected mice could provide information as an indicator on the depletion rates of maternal antibodies and primary antibody response rates in humans.

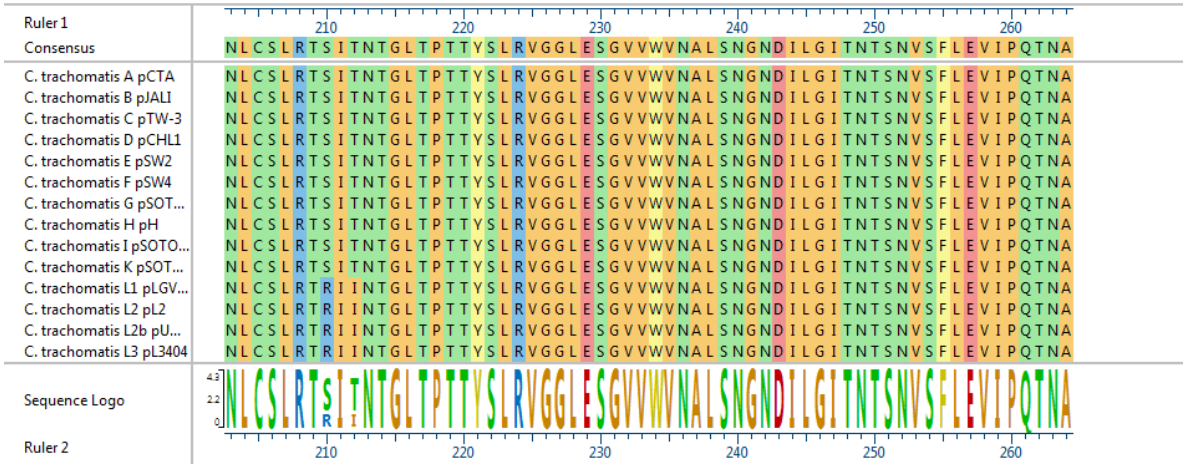
7.3 FINAL CONCLUSIONS

- A sensitive and specific indirect ELISA to detect anti-PGP3 antibodies as an indicator of past and current infection to *C. trachomatis* using a common urogenital derivative of PGP3 was developed.
    - Compared to NAATs, 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.
- For the first time, the seroprevalence rate of anti-PGP3 antibodies within serum samples collected from 13-15-year-old children in England has been determined using a properly designed systematic survey:
    - Overall seroprevalence in 13-, 14- and 15-year-olds from all combined serum sources was 6.87%, 6.70% and 10.47%, respectively.
    - The seroprevalence of antibodies to PGP3 in 15-year-olds found in this study support the antibody prevalence rates of 6-18% found in 17-18-year-olds (165) reported by Horner *et al.*(2013).

APPENDIX I: *CHLAMYDIA TRACHOMATIS* CDS5 AMINO ACID SEQUENCE ALIGNMENTS




Ruler 1
Consensus
C. trachomatis A pCTA
C. trachomatis B pJALI
C. trachomatis C pTW-3
C. trachomatis D pCHL1
C. trachomatis E pSW2
C. trachomatis F pSW4
C. trachomatis G pSOT...
C. trachomatis H pH
C. trachomatis I pSOTO...
C. trachomatis K pSOT...
C. trachomatis L1 pLGV...
C. trachomatis L2 pL2
C. trachomatis L2b pU...
C. trachomatis L3 pL3404
Sequence Logo
Ruler 2
140
150
160
170
180
190
200


Ruler 1
Consensus
C. trachomatis A pCTA
C. trachomatis B pJALI
C. trachomatis C pTW-3
C. trachomatis D pCHL1
C. trachomatis E pSW2
C. trachomatis F pSW4
C. trachomatis G pSOT...
C. trachomatis H pH
C. trachomatis I pSOTO...
C. trachomatis K pSOT...
C. trachomatis L1 pLGV...
C. trachomatis L2 pL2
C. trachomatis L2b pU...
C. trachomatis L3 pL3404
Sequence Logo
Ruler 2
210
220
230
240
250
260

APPENDIX II: PATIENT SERA DETAILS

DETAILS OF PATIENT SERA USED FOR OPTIMISATION, DEVELOPMENT AND EVALUATION OF HIS- AND GST-TAGGED PGP3 AND CLEAVED PGP3 ELISAS (CHAPTERS 4-5)

[a] = Date specifies when previous *C. trachomatis* infection was diagnosed by NAAT at Southampton General Hospital (month/year); [b] = *C. trachomatis* status at time of serum collection as diagnosed by NAAT. (**Note:** all serum collected and received 11/2013-05/2015). Serum samples from non-GUM patients (C1-236) were unrelated to the GUM clinic and these samples were tested for unrelated conditions (Note: patients were not tested for *C. trachomatis* infection and status is unknown). All are under 18-years-old. [c] = Non-GUM sera no. 221 and 234 reacted with GST-PGP3 SW2 in an ELISA and Western blot (see chapter four).

Adult Serum No.	NAAT Pos/Neg[b]	Sex	Age	Previous diagnoses and tests[a]	Code/ group
1	P	F	24	6/2011 NEG	**1**
2	P	M	30	5/2007, 1/2008, 1/2013 ALL NEG	**2**
3	P	M	19		**2**
4	P	F	22	10/2012 NEG	**1**
5	N	M	25	5/2007, 2/2009, 11/2011, 1/2012 ALL NEG	**3**
6	N	F	27		**3**
7	N	F	19	10/2013 POS	**3**
8	N	F	25	4/2011, 12/2009, 8/2008, 3/2008 ALL NEG	**3**
9	N	F	25		**3**
10	N	M	21	10/2011 NEG	**3**
11	N	M	23		**3**
12	N	M	30		**3**
13	N	F	22		**3**
14	N	F	21	6/2012 POS	**3**
15	P	F	31	10/2012 POS	**1**
16	P	M	22		**2**
17	P	F	19		**1**
18	P	F	19		**1**
19	P	F	17		**1**
20	P	M	23		**2**
21	P	M	20		**2**
22	P	F	20		**1**
23	P	M	24		**2**
24	P	F	19		**1**
25	P	F	19		**1**
26	P	M	29		**2**
27	N	F	18	2/2011 NEG	**3**
28	N	F	23	9/2005 NEG, 10/2010 POS	**3**
29	N	M	23		**3**
30	N	F	23		**3**

31	N	F	29		**3**
32	N	F	24	6/2011 NEG	**3**
33	N	M	22		**3**
34	N	F	21	5/2011 NEG	**3**
35	N	F	25	11/2012 NEG	**3**
36	P	F	22		**1**
37	P	F	20		**1**
38	P	F	20		**1**
39	P	F	19		**1**
40	P	F	20		**1**
41	P	F	23	8/2011 NEG	**1**
42	P	M	22	10/2008 NEG	**2**
43	N	M	22	11/2011 NEG	**3**
44	N	F	24	8/2013 NEG	**3**
45	N	F	21	4/2013 NEG	**3**
46	N	M	24		**3**
47	N	F	26		**3**
48	N	F	23		**3**
49	N	F	28		**3**
50	N	M	25		**3**
51	N	F	21		**3**
52	N	F	21		**3**
53	N	F	22		**3**
54	N	F	28		**3**
55	N	M	18	8/2011 NEG	**3**
56	N	F	22	8/2011 NEG	**3**
57	P	F	20	2/2012 NEG	**1**
58	N	M	26		**3**
59	N	F	30		**3**
60	N	M	23		**3**
61	N	F	27		**3**
62	P	M	19		**2**
63	P	F	21	8/2012 NEG	**1**
64	P	F	15		**1**
65	N	F	25		**3**
66	P	F	21	5/2012 NEG	**1**
67	P	M	23	5/2011 NEG	**2**
68	P	M	21	2/2012 NEG	**2**
69	N	F	29	8/2013 NEG	**3**
70	P	F	19		**1**
71	P	F	20		**1**
72	P	F	18		**1**
73	P	M	17		**2**

74	P	F	21		**1**
75	P	M	20		**2**
76	P	F	17		**1**
77	P	M	28		**2**
78	P	M	18		**2**
79	P	F	28		**1**
80	P	M	21		**2**
81	P	F	22	5/2011 NEG	**1**
82	P	M	18		**2**
83	P	M	24		**2**
84	P	F	20		**1**
85	P	M	21		**2**
86	P	F	21		**1**
87	P	F	19		**1**
88	P	F	23	5/2012 NEG	**1**
89	P	M	22	8/2013 NEG	**2**
90	P	F	21	8/2013 NEG	**1**
91	P	M	22		**2**
92	P	F	17		**1**
93	P	F	24	3/2010 POS	**1**
94	P	F	16		**1**
95	P	F	19		**1**
96	P	M	26		**2**
97	P	F	15	**DELETED: DUPLICATE OF NO.64**	
98	P	M	19		**2**
99	P	M	20		**2**
100	P	F	19	10/2013 NEG	**1**
101	P	F	19		**1**
102	P	M	19		**2**
103	P	F	20		**1**
104	P	F	25		**1**
105	P	M	22	8/2011 POS	**2**
106	P	M	20		**2**
107	P	F	19		**1**
108	P	M	21		**2**
109	P	F	19		**1**
110	P	F	23	8/2012 NEG	**1**
111	P	M	18		**2**
112	P	M	18	5/2009 NEG	**2**
113	N	M	28		**3**
114	P	M	27		**2**
115	P	M	19		**2**
116	P	F	22		**1**

117	P	M	19		**2**
118	P	F	22		**1**
119	P	F	18		**1**
120	P	M	19		**2**
121	P	M	30		**2**
122	P	F	21		**1**
123	P	M	20		**2**
124	P	M	19		**2**
125	P	M	30		**2**
126	P	M	20		**2**
127	P	M	22		**2**
128	P	M	29		**2**
129	P	M	20		**2**
130	P	M	26		**2**
131	P	M	24		**2**
132	P	M	21		**2**
133	N	F	15		**3**
134	N	F	18		**3**
135	N	F	23		**3**
136	N	M	21		**3**
137	N	M	23	5/2014 NEG	**3**
138	N	M	22		**3**
139	N	F	22		**3**
140	P	M	23		**2**
141	P	F	22		**1**
142	P	M	28		**2**
143	P	F	22		**1**
144	P	F	22		**1**
145	P	F	27	4/2014 NEG	**1**
146	P	M	24		**2**
147	P	F	22		**1**
148	P	M	31	6/2011, 6/2013 both POS	**2**
149	P	M	23	6/2013, 2/2013, 2/2013 both POS	**2**
150	P	F	21	4/2012, 6/2013 both POS	**1**
151	P	F	18	7/2011, 1/2012, 6/2012, 11/2012 all POS	**1**
152	P	F	21	6/2012, 2/2013 both POS	**1**
153	P	F	26	3/2010 POS	**1**
154	P	F	20	3/2011 POS	**1**
155	P	F	21	9/2011 POS	**1**
156	P	M	20	2/2013 POS	**2**
157	P	M	24	3/2013 POS	**2**
158	N	F	17		**3**
159	N	F	19		**3**

160	N	F	18		**3**
161	N	M	18		**3**
162	N	F	18		**3**
163	N	F	18		**3**
164	N	F	19		**3**
165	N	F	20		**3**
166	N	M	21		**3**
167	N	M	22		**3**
201	P	F	30		**1**
202	P	M	31	09/2014	**2**
203	P	F	19		**1**
204	P	M	21		**2**
205	P	F	21		**1**
206	P	M	23	04/2015	**2**
207	N	F	18		**3**
208	N	F	19		**3**
209	N	F	23		**3**
210	N	F	20		**3**
211	N	M	23		**3**
212	N	F	19		**3**
237	P	F	30		**1**

Non-GUM Serum no.	**Sex**
C1	F
C2	M
C3	F
C4	F
C5	F
C6	F
C7	M
C8	M
213	M
214	M
215	M
216	M
217	M
218	M
219	M
220	F
221[c]	F
222	M
223	M

224	M
225	M
226	F
227	M
228	M
229	M
230	M
231	M
232	F
233	M
234[c]	F
235	F
236	M

APPENDIX III: ELISA VALIDATION DATA

RAW AND BACKGROUND-CORRECTED GST-PGP3 SW2/LGV440 AND PGP3 SW2 ELISA EVALUATION DATA (CHAPTER FIVE).

Note: Serum nos. 9, 11, 28, 46, 58 and 61 were collected from *C. trachomatis* NAAT negative patients but were considered positive according to previous GST-PGP3 ELISAs and Western blots and were not assayed in the PGP3 ELISA. No 148 and 151 were not assayed in the PGP3 assay as there was insufficient sera. Experiments were conducted using GST-PGP3 SW2 batches no 2 and 3.

RAW DATA

Serum No.	GST-Pgp3 SW2 4T1 B2/ B3 (11/2014)		GST-Pgp3 LGV440 4T1 B2 (11/2014)		Cleaved Pgp3 SW2 6P1 B2/ B3 (06/2015)		GST 4T1 B2 (11/2014)		No antigen (11/2014)	
1	3.5	3.5	3.5	3.5	3.5	3.5	0.79	0.808	0.671	0.762
2	0.932	0.898	0.878	0.861	1.566	1.643	0.381	0.416	0.396	0.462
3	0.886	0.881	0.816	0.796	1.876	1.908	0.612	0.622	0.498	0.548
4	0.866	0.869	0.885	0.901	1.723	1.634	0.699	0.725	0.631	0.647
5	0.624	0.638	0.614	0.652	1.324	1.258	0.337	0.35	0.338	0.364
6	1.018	1.027	0.829	0.908	1.436	1.618	0.538	0.513	0.499	0.558
7	0.495	0.555	0.336	0.36	0.791	0.885	0.308	0.299	0.255	0.313
8	0.529	0.611	0.483	0.451	1.048	1.02	0.352	0.384	0.431	0.48
9	2.757	2.756	2.527	2.782	N/A		0.436	0.408	0.368	0.534
10	0.556	0.589	0.484	0.547	1.492	1.455	0.474	0.451	0.504	0.587
11	2.304	2.273	1.895	1.909	N/A		0.434	0.434	0.389	0.487
12	0.304	0.318	0.299	0.315	0.532	0.493	0.245	0.24	0.287	0.324
13	1.204	1.15	1.152	1.254	1.257	1.174	0.878	0.869	0.802	0.812
14	0.554	0.505	0.486	0.534	0.79	0.743	0.349	0.344	0.34	0.36
15	2.754	2.57	2.21	2.38	2.772	2.894	0.47	0.502	0.407	0.419
16	1.337	1.213	0.78	0.765	1.861	1.903	0.417	0.413	0.405	0.418
17	2.505	2.372	2.369	2.22	2.493	2.611	0.496	0.512	0.537	0.578
18	3.5	3.5	3.5	3.5	3.5	3.5	0.508	0.505	0.398	0.414
19	3.5	3.5	3.5	3.39	3.5	3.39	0.362	0.355	0.33	0.356
20	0.869	0.85	0.878	0.878	1.383	1.313	0.684	0.694	0.62	0.606
21	0.549	0.526	0.439	0.417	0.762	0.743	0.287	0.315	0.309	0.325
22	0.684	0.676	0.585	0.57	1.093	1.046	0.398	0.428	0.33	0.332
23	1.791	1.681	1.62	1.614	2.184	2.106	0.471	0.423	0.481	0.524
24	0.855	0.833	0.714	0.671	1.62	1.545	0.436	0.478	0.431	0.399

25	1.196	1.193	1.281	1.125	1.544	1.506	0.979	1.004	1.047	1.122
26	3.5	3.5	3.5	3.493	3.5	3.5	0.443	0.464	0.456	0.512
27	0.81	0.813	0.825	0.797	1.889	1.719	0.556	0.577	0.397	0.411
28	2.752	2.587	2.343	2.428	N/A		0.591	0.623	0.655	0.692
29	0.465	0.452	0.427	0.406	1.136	1.062	0.242	0.277	0.291	0.32
30	0.78	0.738	1.058	0.986	2.037	2.03	0.499	0.452	0.471	0.474
31	0.674	0.629	0.612	0.554	1.092	1.073	0.378	0.399	0.447	0.481
32	0.433	0.396	0.412	0.412	0.573	0.596	0.195	0.219	0.247	0.25
33	0.507	0.493	0.429	0.406	0.792	0.809	0.257	0.289	0.301	0.322
34	0.92	0.832	0.907	0.888	1.374	1.38	0.655	0.681	0.726	0.75
35	0.61	0.555	0.545	0.476	1.273	1.285	0.282	0.307	0.355	0.37
36	2.022	1.933	1.854	1.742	2.105	2.135	0.526	0.555	0.547	0.603
37	2.889	2.911	2.673	2.706	2.793	3.049	0.357	0.367	0.38	0.453
38	1.472	1.478	1.402	1.408	1.659	1.637	0.386	0.396	0.455	0.506
39	3.5	3.5	3.5	3.5	3.5	3.5	0.369	0.374	0.517	0.597
40	0.411	0.431	0.357	0.383	0.66	0.663	0.249	0.258	0.329	0.363
41	3.5	3.5	3.5	3.5	3.5	3.5	0.224	0.238	0.236	0.256
42	1.003	0.978	0.844	0.871	1.327	1.289	0.205	0.229	0.222	0.251
43	0.882	0.853	0.668	0.614	0.842	0.817	0.202	0.217	0.308	0.348
44	0.675	0.689	0.82	0.729	1.191	1.139	0.443	0.46	0.443	0.479
45	0.713	0.695	0.682	0.661	1.341	1.283	0.354	0.527	0.462	0.516
46	3.048	3.077	2.975	2.87	N/A		0.389	0.362	0.288	0.33
47	0.602	0.587	0.578	0.602	0.947	0.903	0.384	0.371	0.359	0.387
48	0.495	0.505	0.448	0.464	0.753	0.761	0.548	0.587	0.486	0.554
49	1.938	1.954	2.292	2.248	1.323	1.254	1.01	1.743	1.289	1.315
50	0.722	0.985	0.914	0.909	1.485	1.477	0.554	0.518	0.487	0.541
51	0.712	0.715	0.569	0.555	0.971	0.991	0.421	0.418	0.395	0.423
52	0.933	0.88	0.916	0.929	1.426	1.461	0.462	0.448	0.408	0.405
53	0.542	0.539	0.469	0.496	0.545	0.538	0.521	0.536	0.297	0.324
54	0.756	0.748	0.538	0.524	0.793	0.754	0.395	0.384	0.381	0.387
55	0.561	0.584	0.662	0.553	0.661	0.655	0.341	0.348	0.308	0.32
56	0.955	0.988	1.45	1.461	1.058	1.05	0.729	0.692	0.822	0.873
57	1.028	0.956	0.427	0.43	1.089	1.08	0.286	0.309	0.326	0.338
58	2.864	2.806	1.11	1.089	N/A		0.602	0.56	0.552	0.587
59	1.295	1.257	1.307	1.297	1.18	1.135	1.025	1.076	0.814	0.842

60	0.602	0.62	0.643	0.638	0.638	0.686	0.477	0.442	0.43	0.421
61	3.295	3.278	3.133	3.094	N/A		0.494	0.525	0.377	0.388
62	0.457	0.472	0.463	0.491	0.653	0.707	0.369	0.371	0.275	0.3
63	3.5	3.297	3.4	3.263	2.919	2.908	0.788	0.814	0.73	0.778
64	0.374	0.383	0.414	0.448	0.753	0.731	0.206	0.224	0.165	0.17
65	1.154	1.216	1.089	1.16	1.141	1.13	0.831	0.895	0.815	0.885
66	0.725	0.712	0.695	0.708	1.024	1.02	0.575	0.624	0.576	0.612
67	3.38	3.241	3.299	3.168	3.162	3.002	0.467	0.45	0.422	0.433
68	0.334	0.303	0.334	0.322	0.619	0.567	0.225	0.227	0.163	0.175
69	0.795	0.831	0.714	0.699	0.945	0.991	0.478	0.497	0.506	0.537
70	0.53	0.574	0.514	0.473	0.713	0.706	0.396	0.428	0.373	0.371
71	0.829	0.707	0.608	0.582	1.339	1.377	0.373	0.415	0.322	0.347
72	0.891	0.846	0.874	0.834	1.527	1.559	0.894	0.953	0.43	0.454
73	1.005	0.997	1.291	1.257	1.303	1.278	0.884	0.877	0.532	0.573
74	2.919	2.782	2.733	2.648	2.789	2.756	0.425	0.472	0.281	0.323
75	0.738	0.74	0.775	0.803	1.07	1.093	0.594	0.577	0.341	0.407
76	3.5	3.5	3.5	3.5	3.5	3.5	0.958	0.982	0.599	0.658
77	0.801	0.748	0.729	0.714	1.411	1.388	0.389	0.415	0.376	0.407
78	1.949	1.933	1.694	1.657	1.869	1.825	0.436	0.465	0.348	0.381
79	2.939	3.067	2.849	2.665	3.347	3.187	0.563	0.558	0.409	0.406
80	1.905	1.758	1.624	1.584	2.119	2.049	0.361	0.392	0.255	0.297
81	3.333	3.21	3.24	3.144	3.359	3.285	0.458	0.474	0.405	0.426
82	3.081	2.915	1.996	1.973	3.067	2.939	0.325	0.355	0.277	0.294
83	2.594	2.587	2.442	2.338	2.619	2.539	0.416	0.431	0.301	0.321
84	3.41	3.395	3.272	3.259	3.123	3.334	0.507	0.515	0.319	0.377
85	1.868	1.704	1.131	1.106	2.077	2.097	0.935	0.884	0.517	0.528
86	3.03	2.955	2.932	2.953	2.745	2.683	0.535	0.562	0.443	0.469
87	2.94	2.929	2.495	2.448	2.425	2.32	0.618	0.628	0.589	0.767
88	3.5	3.5	3.5	3.5	3.5	3.5	0.263	0.275	0.139	0.146
89	0.706	0.661	0.66	0.67	1.066	1.095	0.448	0.469	0.339	0.362
90	3.139	3.037	2.846	3.064	2.608	2.592	0.567	0.654	0.454	0.453
91	0.648	0.636	0.69	0.684	1.073	1.057	0.386	0.401	0.362	0.398
92	1.082	1.02	0.981	0.991	1.474	1.41	0.765	0.753	0.611	0.603
93	3.5	3.451	3.44	3.413	3.205	3.241	0.877	0.876	0.862	0.815
94	2.876	2.724	1.401	1.41	3.101	3.089	0.315	0.291	0.198	0.219

95	0.807	0.786	0.85	0.81	1.135	1.067	0.365	0.432	0.4	0.366
96	3.5	3.5	3.486	3.5	3.5	3.361	0.484	0.521	0.348	0.381
97										
98	0.676	0.673	0.839	0.808	0.644	0.616	0.446	0.488	0.489	0.476
99	0.853	0.818	0.901	0.904	0.831	0.865	0.698	0.729	0.724	0.769
100	0.494	0.516	0.45	0.438	0.422	0.419	0.38	0.381	0.441	0.484
101	3.134	3.009	3.064	3.051	2.44	2.489	0.544	0.556	0.362	0.397
102	3.484	3.159	2.053	2.042	3.336	3.376	0.456	0.482	0.421	0.372
103	3.5	3.468	3.5	3.5	3.369	3.414	0.606	0.623	0.589	0.577
104	3.5	3.5	3.167	3.26	3.5	3.5	1.24	1.218	1.399	1.409
105	3.405	3.404	3.269	3.359	3.218	3.146	0.986	1.031	0.813	0.809
106	0.729	0.754	0.75	0.722	0.873	0.829	0.594	0.514	0.497	0.542
107	0.745	0.722	0.85	0.818	1.203	1.201	0.371	0.374	0.365	0.388
108	0.638	0.638	0.577	0.604	0.947	0.927	0.456	0.424	0.423	0.414
109	3.5	3.5	3.5	3.5	3.5	3.458	0.559	0.508	0.497	0.468
110	2.745	2.694	2.065	2.025	2.065	2.56	0.62	0.606	0.498	0.495
111	2.827	2.708	1.963	2.107	2.874	2.107	0.575	0.598	0.493	0.359
112	3.309	3.5	3.5	3.5	3.5	3.5	0.801	0.823	0.656	0.702
113	3.5	3.5	3.5	3.5	3.5	3.5	0.405	0.405	0.358	0.385
114	2.717	2.604	2.464	2.474	2.456	2.541	0.399	0.402	0.412	0.425
115	1.082	1.061	0.943	0.959	1.113	1.21	0.767	0.71	0.751	0.792
116	3.341	3.325	3.085	3.163	3.456	3.496	0.669	0.624	0.614	0.604
117	1.176	1.059	1.054	1.047	1.171	1.251	0.627	0.624	0.57	0.539
118	1.059	1.052	0.941	1.008	1.143	1.272	0.771	0.77	0.624	0.591
119	2.661	2.65	2.038	1.88	2.614	2.561	0.964	0.892	0.486	0.474
120	3.405	3.5	2.822	2.75	2.821	2.754	0.801	0.732	0.66	0.617
121	1.201	1.241	1.084	1.181	1.942	1.821	0.723	0.874	0.825	0.754
122	3.438	3.489	2.318	2.382	3.5	3.5	0.543	0.554	0.322	0.356
123	2.634	2.578	2.41	2.35	2.819	2.733	0.838	0.916	0.815	0.808
124	2.558	2.543	2.431	2.481	2.797	2.88	1.311	1.336	1.303	1.339
125	2.207	2.314	1.93	1.943	2.461	2.532	0.662	0.673	0.547	0.588
126	1.032	1.036	1.11	1.113	1.671	1.675	0.86	0.848	0.789	0.834
127	3.5	3.5	3.5	3.5	3.5	3.5	0.899	0.953	0.695	0.721
128	1.604	1.505	1.569	1.57	2.346	2.484	0.99	1.02	0.845	0.889
129	1.667	1.577	1.565	1.61	2.286	2.319	0.565	0.533	0.42	0.46

130	3.347	3.381	3.303	3.127	3.5	3.5	0.472	0.452	0.409	0.423
131	3.5	3.5	3.5	3.5	3.5	3.5	0.952	0.881	0.828	0.816
132	1.613	1.493	1.299	1.312	1.81	1.841	0.833	0.761	0.64	0.644
133	1.259	1.212	1.016	1.049	1.753	1.767	0.771	0.767	0.832	0.868
134	1.012	0.985	0.921	1.006	1.297	1.498	0.724	0.74	0.719	0.812
135	1.16	1.097	1.276	1.314	1.602	1.599	0.794	0.809	0.794	0.861
136	0.674	0.65	0.58	0.605	0.897	0.923	0.458	0.468	0.488	0.512
137	1.449	1.416	1.186	1.149	1.997	1.918	0.634	0.635	0.659	0.668
138	1.019	1.008	0.944	0.918	1.527	1.509	0.657	0.673	0.642	0.704
139	1.368	1.304	1.23	1.272	1.885	1.778	1.1	1.045	1.134	1.189
140	3.18	2.247	3.17	3.178	3.395	3.334	0.67	0.69	0.529	0.513
141	3.072	3.14	2.609	2.638	3.225	3.102	1.281	1.197	1.135	1.237
142	3.5	3.5	3.5	3.5	3.5	3.5	0.954	0.932	0.766	0.816
143	0.784	0.747	0.82	0.799	1.266	1.195	0.638	0.648	0.556	0.553
144	3.5	3.5	3.5	3.5	3.5	3.5	1.464	1.345	0.98	1.034
145	3.5	3.5	3.5	3.5	3.5	3.5	1.303	1.351	1.153	1.267
146	2.859	2.818	2.921	2.943	2.84	3.018	0.633	0.638	0.614	0.633
147	3.5	3.5	2.705	2.281	3.5	3.5	1.194	1.197	0.844	1.003
148	3.5	3.5	3.098	3.166	N/A		0.404	0.378	0.266	0.272
149	2.052	1.904	1.576	1.557	2.015	2.133	0.787	0.744	0.691	0.755
150	3.5	3.5	3.5	3.5	3.5	3.5	1.87	2.024	1.438	1.522
151	1.948	1.954	1.505	1.503	N/A		1.096	1.033	0.943	0.951
152	2.154	1.977	1.642	1.718	2.334	2.34	0.953	0.939	0.688	0.72
153	3.5	3.5	3.5	3.5	3.5	3.5	0.923	0.918	0.906	0.902
154	3.5	3.313	3.404	3.209	3.5	3.473	1.008	0.964	0.926	0.99
155	2.395	2.221	1.932	1.829	2.777	2.712	0.918	0.899	0.866	0.864
156	2.816	2.966	2.602	2.47	2.497	2.743	0.821	0.764	0.74	0.865
157	1.522	1.352	1.087	1.147	1.594	1.415	0.71	0.678	0.669	0.729
158	1.556	1.641	1.2	1.101	0.785	0.745	1.664	1.418	1.107	1.119
159	0.848	0.83	0.78	0.698	0.742	0.66	0.842	0.734	0.701	0.634
160	1.369	1.312	1.123	1.23	1.087	1.029	1.129	1.119	1.113	1.18
161	0.853	0.885	0.87	0.789	0.594	0.622	0.713	0.628	0.64	0.695
162	1.094	1.192	1.001	0.987	0.864	0.921	0.817	0.834	0.757	0.846
163	1.4	1.455	0.989	1.003	1.105	1.031	1.071	1.102	0.925	1.019
164	0.962	1.017	0.765	0.729	1.097	1.035	0.635	0.656	0.639	0.657

165	1.051	1.066	0.825	0.73	0.889	0.808	0.724	0.823	0.684	0.728
166	1.075	1.082	0.999	1.01	0.677	0.633	0.783	0.816	0.983	0.994
167	0.903	0.87	0.769	0.989	0.693	0.697	0.601	0.657	0.68	0.632
201	N/A		N/A		0.493	0.51	N/A		N/A	
202					1.847	1.905				
203					2.542	2.428				
204					3.5	3.433				
205					1.632	1.713				
206					1.742	1.794				
207					1.613	1.522				
208					0.654	0.67				
209					0.864	0.888				
210					1.006	1.014				
211					0.958	0.957				
212					1.108	1.098				
237					2.239	2.168				

BACKGROUND-CORRECTED DATA

Serum No.	**GST-Pgp3 SW2 4T1 B2/B3 (11/2014)**		**GST-Pgp3 LGV440 4T1 (11/2014)**		**Cleaved Pgp3 SW2 6P1 (06/2015)**		**GST 4T1 B2 (11/2014)**	
1	2.7835	2.7835	2.7835	2.7835	2.9385	2.9385	0.0735	0.0915
2	0.503	0.469	0.449	0.432	1.1355	1.2125	-0.048	-0.013
3	0.363	0.358	0.293	0.273	1.393	1.425	0.089	0.099
4	0.227	0.23	0.246	0.262	1.0385	0.9495	0.06	0.086
5	0.273	0.287	0.263	0.301	0.782	0.716	-0.014	-0.001
6	0.4895	0.4985	0.3005	0.3795	0.89	1.072	0.0095	-0.0155
7	0.211	0.271	0.052	0.076	0.5145	0.6085	0.024	0.015
8	0.0735	0.1555	0.0275	-0.0045	0.674	0.646	-0.1035	-0.0715
9	2.306	2.305	2.076	2.331	N/A		-0.015	-0.043
10	0.0105	0.0435	-0.0615	0.0015	0.913	0.876	-0.0715	-0.0945
11	1.866	1.835	1.457	1.471	N/A		-0.004	-0.004
12	-0.0015	0.0125	-0.0065	0.0095	0.224	0.185	-0.0605	-0.0655
13	0.397	0.343	0.345	0.447	0.3485	0.2655	0.071	0.062
14	0.204	0.155	0.136	0.184	0.4825	0.4355	-0.001	-0.006
15	2.341	2.157	1.797	1.967	2.3425	2.4645	0.057	0.089

16	0.9255	0.8015	0.3685	0.3535	1.494	1.536	0.0055	0.0015
17	1.9475	1.8145	1.8115	1.6625	2.0935	2.2115	-0.0615	-0.0455
18	3.094	3.094	3.094	3.094	3.233	3.233	0.102	0.099
19	3.157	3.157	3.157	3.047	3.01	2.9	0.019	0.012
20	0.256	0.237	0.265	0.265	0.9455	0.8755	0.071	0.081
21	0.232	0.209	0.122	0.1	0.4945	0.4755	-0.03	-0.002
22	0.353	0.345	0.254	0.239	0.7835	0.7365	0.067	0.097
23	1.2885	1.1785	1.1175	1.1115	1.884	1.806	-0.0315	-0.0795
24	0.44	0.418	0.299	0.256	0.989	0.914	0.021	0.063
25	0.1115	0.1085	0.1965	0.0405	0.406	0.368	-0.1055	-0.0805
26	3.016	3.016	3.016	3.009	3.067	3.067	-0.041	-0.02
27	0.406	0.409	0.421	0.393	1.2815	1.1115	0.152	0.173
28	2.0785	1.9135	1.6695	1.7545	N/A		-0.0825	-0.0505
29	0.1595	0.1465	0.1215	0.1005	0.6885	0.6145	-0.0635	-0.0285
30	0.3075	0.2655	0.5855	0.5135	1.721	1.714	0.0265	-0.0205
31	0.21	0.165	0.148	0.09	0.521	0.502	-0.086	-0.065
32	0.1845	0.1475	0.1635	0.1635	0.164	0.187	-0.0535	-0.0295
33	0.1955	0.1815	0.1175	0.0945	0.3915	0.4085	-0.0545	-0.0225
34	0.182	0.094	0.169	0.15	1.01	1.016	-0.083	-0.057
35	0.2475	0.1925	0.1825	0.1135	0.898	0.91	-0.0805	-0.0555
36	1.447	1.358	1.279	1.167	1.528	1.558	-0.049	-0.02
37	2.4725	2.4945	2.2565	2.2895	2.305	2.561	-0.0595	-0.0495
38	0.9915	0.9975	0.9215	0.9275	1.408	1.386	-0.0945	-0.0845
39	2.943	2.943	2.943	2.943	3.112	3.112	-0.188	-0.183
40	0.065	0.085	0.011	0.037	0.5285	0.5315	-0.097	-0.088
41	3.254	3.254	3.254	3.254	3.233	3.233	-0.022	-0.008
42	0.7665	0.7415	0.6075	0.6345	1.03	0.992	-0.0315	-0.0075
43	0.554	0.525	0.34	0.286	0.586	0.561	-0.126	-0.111
44	0.214	0.228	0.359	0.268	0.9075	0.8555	-0.018	-0.001
45	0.224	0.206	0.193	0.172	0.976	0.918	-0.135	0.038
46	2.739	2.768	2.666	2.561	N/A		0.08	0.053
47	0.229	0.214	0.205	0.229	0.6245	0.5805	0.011	-0.002
48	-0.025	-0.015	-0.072	-0.056	0.15	0.158	0.028	0.067
49	0.636	0.652	0.99	0.946	0.9065	0.8375	-0.292	0.441
50	0.208	0.471	0.4	0.395	1.2615	1.2535	0.04	0.004

51	0.303	0.306	0.16	0.146	0.5785	0.5985	0.012	0.009
52	0.5265	0.4735	0.5095	0.5225	1.1525	1.1875	0.0555	0.0415
53	0.2315	0.2285	0.1585	0.1855	0.2205	0.2135	0.2105	0.2255
54	0.372	0.364	0.154	0.14	0.461	0.422	0.011	0
55	0.247	0.27	0.348	0.239	0.432	0.426	0.027	0.034
56	0.1075	0.1405	0.6025	0.6135	0.255	0.247	-0.1185	-0.1555
57	0.696	0.624	0.095	0.098	0.705	0.696	-0.046	-0.023
58	2.2945	2.2365	0.5405	0.5195	N/A		0.0325	-0.0095
59	0.467	0.429	0.479	0.469	0.4055	0.3605	0.197	0.248
60	0.1765	0.1945	0.2175	0.2125	0.122	0.17	0.0515	0.0165
61	2.9125	2.8955	2.7505	2.7115	N/A		0.1115	0.1425
62	0.1695	0.1845	0.1755	0.2035	0.4195	0.4735	0.0815	0.0835
63	2.746	2.543	2.646	2.509	2.474	2.463	0.034	0.06
64	0.2065	0.2155	0.2465	0.2805	0.551	0.529	0.0385	0.0565
65	0.304	0.366	0.239	0.31	0.267	0.256	-0.019	0.045
66	0.131	0.118	0.101	0.114	0.1005	0.0965	-0.019	0.03
67	2.9525	2.8135	2.8715	2.7405	2.7385	2.5785	0.0395	0.0225
68	0.165	0.134	0.165	0.153	0.4355	0.3835	0.056	0.058
69	0.2735	0.3095	0.1925	0.1775	0.6005	0.6465	-0.0435	-0.0245
70	0.158	0.202	0.142	0.101	0.387	0.38	0.024	0.056
71	0.4945	0.3725	0.2735	0.2475	0.9065	0.9445	0.0385	0.0805
72	0.449	0.404	0.432	0.392	1.237	1.269	0.452	0.511
73	0.4525	0.4445	0.7385	0.7045	0.926	0.901	0.3315	0.3245
74	2.617	2.48	2.431	2.346	2.2465	2.2135	0.123	0.17
75	0.364	0.366	0.401	0.429	0.468	0.491	0.22	0.203
76	2.8715	2.8715	2.8715	2.8715	2.897	2.897	0.3295	0.3535
77	0.4095	0.3565	0.3375	0.3225	0.983	0.96	-0.0025	0.0235
78	1.5845	1.5685	1.3295	1.2925	0.8545	0.8105	0.0715	0.1005
79	2.5315	2.6595	2.4415	2.2575	2.822	2.662	0.1555	0.1505
80	1.629	1.482	1.348	1.308	1.8655	1.7955	0.085	0.116
81	2.9175	2.7945	2.8245	2.7285	2.9505	2.8765	0.0425	0.0585
82	2.7955	2.6295	1.7105	1.6875	2.7825	2.6545	0.0395	0.0695
83	2.283	2.276	2.131	2.027	2.1805	2.1005	0.105	0.12
84	3.062	3.047	2.924	2.911	2.814	3.025	0.159	0.167
85	1.3455	1.1815	0.6085	0.5835	1.737	1.757	0.4125	0.3615

86	2.574	2.499	2.476	2.497	2.4875	2.4255	0.079	0.106
87	2.262	2.251	1.817	1.77	2.18	2.075	-0.06	-0.05
88	3.3575	3.3575	3.3575	3.3575	3.2855	3.2855	0.1205	0.1325
89	0.3555	0.3105	0.3095	0.3195	0.3975	0.4265	0.0975	0.1185
90	2.6855	2.5835	2.3925	2.6105	2.2565	2.2405	0.1135	0.2005
91	0.268	0.256	0.31	0.304	0.7915	0.7755	0.006	0.021
92	0.475	0.413	0.374	0.384	0.8905	0.8265	0.158	0.146
93	2.6615	2.6125	2.6015	2.5745	2.46	2.496	0.0385	0.0375
94	2.6675	2.5155	1.1925	1.2015	2.948	2.936	0.1065	0.0825
95	0.424	0.403	0.467	0.427	0.923	0.855	-0.018	0.049
96	3.1355	3.1355	3.1215	3.1355	3.1885	3.0495	0.1195	0.1565
97	N/A							
98	0.1935	0.1905	0.3565	0.3255	0.4125	0.3845	-0.0365	0.0055
99	0.1065	0.0715	0.1545	0.1575	0.4295	0.4635	-0.0485	-0.0175
100	0.0315	0.0535	-0.0125	-0.0245	0.238	0.235	-0.0825	-0.0815
101	2.7545	2.6295	2.6845	2.6715	2.228	2.277	0.1645	0.1765
102	3.0875	2.7625	1.6565	1.6455	2.9895	3.0295	0.0595	0.0855
103	2.917	2.885	2.917	2.917	2.7515	2.7965	0.023	0.04
104	2.096	2.096	1.763	1.856	2.9035	2.9035	-0.164	-0.186
105	2.594	2.593	2.458	2.548	2.837	2.765	0.175	0.22
106	0.2095	0.2345	0.2305	0.2025	0.61	0.566	0.0745	-0.0055
107	0.3685	0.3455	0.4735	0.4415	0.6565	0.6545	-0.0055	-0.0025
108	0.2195	0.2195	0.1585	0.1855	0.76	0.74	0.0375	0.0055
109	3.0175	3.0175	3.0175	3.0175	2.942	2.9	0.0765	0.0255
110	2.2485	2.1975	1.5685	1.5285	1.575	2.07	0.1235	0.1095
111	2.401	2.282	1.537	1.681	2.408	1.641	0.149	0.172
112	2.63	2.821	2.821	2.821	2.86	2.86	0.122	0.144
113	3.1285	3.1285	3.1285	3.1285	3.116	3.116	0.0335	0.0335
114	2.2985	2.1855	2.0455	2.0555	2.0425	2.1275	-0.0195	-0.0165
115	0.3105	0.2895	0.1715	0.1875	0.3005	0.3975	-0.0045	-0.0615
116	2.732	2.716	2.476	2.554	2.851	2.891	0.06	0.015
117	0.6215	0.5045	0.4995	0.4925	0.41	0.49	0.0725	0.0695
118	0.4515	0.4445	0.3335	0.4005	0.503	0.632	0.1635	0.1625
119	2.181	2.17	1.558	1.4	1.798	1.745	0.484	0.412
120	2.7665	2.8615	2.1835	2.1115	1.9975	1.9305	0.1625	0.0935

121	0.4115	0.4515	0.2945	0.3915	0.5285	0.4075	-0.0665	0.0845
122	3.099	3.15	1.979	2.043	2.9655	2.9655	0.204	0.215
123	1.8225	1.7665	1.5985	1.5385	1.3955	1.3095	0.0265	0.1045
124	1.237	1.222	1.11	1.16	0.848	0.931	-0.01	0.015
125	1.6395	1.7465	1.3625	1.3755	1.6915	1.7625	0.0945	0.1055
126	0.2205	0.2245	0.2985	0.3015	0.6105	0.6145	0.0485	0.0365
127	2.792	2.792	2.792	2.792	2.6585	2.6585	0.191	0.245
128	0.737	0.638	0.702	0.703	1.2585	1.3965	0.123	0.153
129	1.227	1.137	1.125	1.17	1.765	1.798	0.125	0.093
130	2.931	2.965	2.887	2.711	2.404	2.404	0.056	0.036
131	2.678	2.678	2.678	2.678	1.998	1.998	0.13	0.059
132	0.971	0.851	0.657	0.67	0.7885	0.8195	0.191	0.119
133	0.409	0.362	0.166	0.199	0.8295	0.8435	-0.079	-0.083
134	0.2465	0.2195	0.1555	0.2405	0.2585	0.4595	-0.0415	-0.0255
135	0.3325	0.2695	0.4485	0.4865	0.41	0.407	-0.0335	-0.0185
136	0.174	0.15	0.08	0.105	0.2035	0.2295	-0.042	-0.032
137	0.7855	0.7525	0.5225	0.4855	1.006	0.927	-0.0295	-0.0285
138	0.346	0.335	0.271	0.245	0.822	0.804	-0.016	0
139	0.2065	0.1425	0.0685	0.1105	-0.01	-0.117	-0.0615	-0.1165
140	2.659	1.726	2.649	2.657	2.448	2.387	0.149	0.169
141	1.886	1.954	1.423	1.452	1.8585	1.7355	0.095	0.011
142	2.709	2.709	2.709	2.709	1.65	1.65	0.163	0.141
143	0.2295	0.1925	0.2655	0.2445	0.471	0.4	0.0835	0.0935
144	2.493	2.493	2.493	2.493	2.478	2.478	0.457	0.338
145	2.29	2.29	2.29	2.29	2.2705	2.2705	0.093	0.141
146	2.2355	2.1945	2.2975	2.3195	1.8065	1.9845	0.0095	0.0145
147	2.5765	2.5765	1.7815	1.3575	2.5355	2.5355	0.2705	0.2735
148	3.231	3.231	2.829	2.897	N/A		0.135	0.109
149	1.329	1.181	0.853	0.834	0.945	1.063	0.064	0.021
150	2.02	2.02	2.02	2.02	2.542	2.542	0.39	0.544
151	1.001	1.007	0.558	0.556	N/A		0.149	0.086
152	1.45	1.273	0.938	1.014	1.1055	1.1115	0.249	0.235
153	2.596	2.596	2.596	2.596	2.6145	2.6145	0.019	0.014
154	2.542	2.355	2.446	2.251	2.429	2.402	0.05	0.006
155	1.53	1.356	1.067	0.964	1.7515	1.6865	0.053	0.034

156	2.0135	2.1635	1.7995	1.6675	1.5855	1.8315	0.0185	-0.0385
157	0.823	0.653	0.388	0.448	0.949	0.77	0.011	-0.021
158	0.443	0.528	0.087	-0.012	0.216	0.176	0.551	0.305
159	0.1805	0.1625	0.1125	0.0305	0.2855	0.2035	0.1745	0.0665
160	0.2225	0.1655	-0.0235	0.0835	0.1835	0.1255	-0.0175	-0.0275
161	0.1855	0.2175	0.2025	0.1215	0.0245	0.0525	0.0455	-0.0395
162	0.2925	0.3905	0.1995	0.1855	0.447	0.504	0.0155	0.0325
163	0.428	0.483	0.017	0.031	0.1925	0.1185	0.099	0.13
164	0.314	0.369	0.117	0.081	0.6105	0.5485	-0.013	0.008
165	0.345	0.36	0.119	0.024	0.086	0.005	0.018	0.117
166	0.0865	0.0935	0.0105	0.0215	-0.1715	-0.2155	-0.2055	-0.1725
167	0.247	0.214	0.113	0.333	0.4755	0.4795	-0.055	0.001
201	N/A		N/A		-0.0245	-0.0075	N/A	
202					1.2225	1.2805		
203					2.0165	1.9025		
204					2.9795	2.9125		
205					1.152	1.233		
206					1.129	1.181		
207					0.357	0.266		
208					0.016	0.032		
209					-0.0965	-0.0725		
210					-0.111	-0.103		
211					0.207	0.206		
212					-0.0555	-0.0655		
237					0.675	0.604		

APPENDIX IV: GST-PGP3 ELISA REPRODUCIBILITY DATA

RAW AND BACKGROUND-CORRECTED REPRODUCIBILITY DATA OF GST-PGP3 SW2 ELISA (CHAPTER FIVE)

RAW DATA

Assay no	204				205				234				215			
1	3.414	3.5	3.491	3.371	1.949	1.965	1.698	1.809	1.356	1.347	1.166	1.222	0.511	0.595	0.477	0.46
2	3.336	3.358	3.243	3.254	1.542	1.502	1.423	1.383	1.073	1.059	0.929	0.975	0.437	0.446	0.416	0.444
3	3.133	3.339	3.112	3.174	1.476	1.442	1.186	1.363	0.985	0.973	0.938	0.864	0.482	0.539	0.433	0.156
4	3.207	3.301	3.112	3.187	1.514	1.526	1.476	1.467	1.085	1.018	0.913	0.986	0.425	0.437	0.411	0.481
5	3.268	2.981	3.255	3.258	1.461	1.704	1.635	1.709	1.22	1.179	1.163	1.176	0.549	0.584	0.543	0.576
6	3.367	3.298	3.101	3.182	1.782	1.792	1.532	1.441	1.168	1.128	1.044	1.008	0.504	0.534	0.423	0.43
7	3.5	3.5	3.5	3.5	2.075	2.018	1.932	1.918	1.444	1.425	1.246	1.315	0.579	0.606	0.566	0.234
8	3.376	3.462	3.354	3.339	1.922	1.858	1.618	1.642	1.048	1.12	1.062	1.041	0.562	0.578	0.532	0.553
9	3.418	3.436	3.395	3.443	1.612	1.403	1.417	1.416	1.133	1.132	1.056	1.073	0.516	0.554	0.474	0.51
10	3.473	3.475	3.367	3.434	1.558	1.566	1.467	1.496	1.076	1.117	1.017	0.961	0.48	0.491	0.468	0.461
11	3.345	3.279	3.366	3.328	1.603	1.648	1.666	1.266	1.058	1.027	0.946	0.907	0.475	0.5	0.431	0.472
12	3.122	3	2.904	3.059	1.727	1.592	1.801	1.802	1.088	1.026	1.347	1.313	0.595	0.588	0.778	0.886
13	3.172	3.254	3.369	3.394	2.116	1.63	1.705	1.774	0.922	0.989	1.07	0.973	0.458	0.453	0.507	0.523
14	3.198	3.247	3.236	3.212	1.568	1.476	1.387	1.4	1.038	0.991	0.928	0.924	0.552	0.523	0.53	0.524
15	3.085	3.03	3.018	3.017	1.37	1.321	1.295	1.269	0.934	0.919	0.813	0.793	0.505	0.491	0.478	0.482
16	3.166	3.154	3.154	3.144	1.604	1.509	1.546	1.459	1.093	1.086	1.205	1.148	0.546	0.546	0.542	0.538
17	3.358	3.265	3.22	3.291	1.699	1.697	1.697	1.562	1.09	1.074	1.196	1.119	0.166	0.174	0.173	0.182
18	2.704	2.492	2.771	2.773	1.444	1.354	1.483	1.561	1.035	1.061	1.015	1.082	0.442	0.524	0.515	0.53
19	3.12	3.049	2.873	2.848	1.408	1.358	1.401	1.357	0.963	0.994	0.953	0.951	0.408	0.497	0.41	0.443
20	3.373	3.23	3.124	3.074	1.591	1.576	1.428	1.383	0.841	0.981	1.016	1.033	0.477	0.5	0.54	0.575

Assay no	216				217				anti-Pgp3 pAb				anti-GST mAb			
1	0.542	0.461	0.365	0.354	0.391	0.387	0.283	0.275	3.013	2.87	3.198	3.348	1.784	1.953	1.645	1.357
2	0.329	0.306	0.297	0.28	0.306	0.22	0.247	0.251	2.749	2.795	2.868	2.922	1.333	1.372	1.34	1.367
3	0.236	0.23	0.282	0.273	0.28	0.26	0.299	0.288	3.099	3.025	3.343	3.201	1.56	1.695	1.881	1.847
4	0.355	0.332	0.283	0.28	0.325	0.324	0.329	0.323	3.043	3.146	3.078	3.295	1.296	1.318	1.299	1.432
5	0.381	0.353	0.315	0.288	0.516	0.514	0.452	0.437	3.004	3.232	2.977	3.113	1.329	1.279	1.459	1.493
6	0.462	0.432	0.341	0.313	0.429	0.41	0.338	0.313	2.926	2.903	3.459	3.488	1.814	1.853	1.539	1.646
7	0.432	0.366	0.36	0.35	0.601	0.582	0.532	0.532	3.099	3.012	3.206	3.359	1.99	2.013	1.785	1.856
8	0.401	0.357	0.385	0.356	0.333	0.323	0.319	0.284	2.671	2.78	3.294	3.351	1.672	1.842	2.019	2.059
9	0.296	0.258	0.299	0.34	0.461	0.377	0.325	0.33	2.272	2.556	3.042	3.147	1.443	1.462	1.557	1.524
10	0.322	0.295	0.321	0.288	0.346	0.338	0.354	0.35	3.042	3.042	3.215	3.143	1.748	1.943	1.516	1.677
11	0.178	0.165	0.168	0.159	0.254	0.226	0.214	0.208	3.248	3.148	3.216	3.006	1.846	1.534	1.976	1.004
12	0.381	0.346	0.771	0.654	0.501	0.464	0.777	0.842	2.516	2.698	3.077	3.004	1.405	1.433	1.615	1.809
13	0.284	0.25	0.246	0.236	0.435	0.384	0.415	0.394	2.798	3.405	3.315	3.181	1.357	1.414	1.396	1.453
14	0.366	0.346	0.394	0.365	0.407	0.401	0.428	0.469	3.068	3.06	2.998	2.845	1.405	1.427	1.352	1.444
15	0.224	0.209	0.211	0.197	0.423	0.394	0.366	0.365	2.916	2.854	2.916	2.94	1.317	1.388	1.206	1.378
16	0.355	0.318	0.321	0.295	0.419	0.413	0.471	0.454	3.15	3.041	2.956	3.012	1.526	1.615	1.556	1.611
17	0.457	0.428	0.505	0.49	0.707	0.647	0.696	0.698	3.041	2.939	3.178	3.012	1.556	1.517	1.515	1.634
18	0.414	0.39	0.425	0.39	0.23	0.229	0.248	0.216	2.767	3.021	2.997	2.836	1.948	1.926	1.937	2.049
19	0.374	0.407	0.575	0.512	0.255	0.234	0.395	0.367	3.5	3.5	3.5	3.5	1.737	1.734	1.633	2.01
20	0.583	0.513	0.484	0.469	0.294	0.279	0.226	0.231	3.5	3.5	3.38	2.679	1.638	1.712	1.464	1.655

BACKGROUND-CORRECTED DATA

Assay no	204				205				234				215			
1	3.066	3.152	3.132	3.012	1.636	1.652	1.537	1.648	1.156	1.147	1.046	1.102	0.222	0.306	0.316	0.299
2	3.156	3.178	3.069	3.08	1.407	1.367	1.28	1.24	0.991	0.977	0.845	0.891	0.277	0.286	0.264	0.292
3	2.729	2.935	2.892	2.954	1.161	1.127	1.046	1.223	0.733	0.721	0.846	0.772	0.164	0.221	0.105	-0.17
4	2.944	3.038	2.862	2.937	1.346	1.358	1.313	1.304	0.977	0.91	0.806	0.879	0.246	0.258	0.252	0.322
5	2.953	2.666	2.759	2.762	1.189	1.432	1.362	1.436	1.031	0.99	0.976	0.989	0.341	0.376	0.335	0.368
6	3.055	2.986	2.816	2.897	1.494	1.504	1.29	1.199	1	0.96	0.951	0.915	0.269	0.299	0.266	0.273
7	2.791	2.791	2.91	2.91	1.753	1.696	1.675	1.661	1.246	1.227	1.051	1.12	0.301	0.328	0.137	-0.2
8	3.094	3.18	3.096	3.081	1.729	1.665	1.433	1.457	0.949	1.021	0.967	0.946	0.356	0.372	0.352	0.373
9	3.16	3.178	3.137	3.185	1.433	1.224	1.252	1.251	1.037	1.036	0.947	0.964	0.322	0.36	0.298	0.334
10	3.203	3.205	3.017	3.084	1.369	1.377	1.283	1.312	0.978	1.019	0.912	0.856	0.291	0.302	0.292	0.285
11	3.114	3.048	3.175	3.137	1.478	1.523	1.581	1.181	0.969	0.938	0.866	0.827	0.32	0.345	0.303	0.344
12	2.872	2.75	2.763	2.918	1.463	1.328	1.177	1.178	0.919	0.857	0.845	0.811	0.374	0.367	0.284	0.392
13	2.839	2.921	3.018	3.043	1.896	1.41	1.467	1.536	0.846	0.913	0.985	0.888	0.296	0.291	0.341	0.357
14	2.842	2.891	2.857	2.833	1.367	1.275	1.192	1.205	0.919	0.872	0.762	0.758	0.36	0.331	0.338	0.332
15	2.772	2.717	2.662	2.661	1.152	1.103	1.091	1.065	0.799	0.784	0.695	0.675	0.324	0.31	0.294	0.298
16	2.772	2.76	2.749	2.739	1.359	1.264	1.305	1.218	0.941	0.934	1.033	0.976	0.327	0.327	0.326	0.322
17	3.09	2.997	2.96	3.031	1.378	1.376	1.441	1.306	0.915	0.899	0.994	0.917	0.075	0.083	0.076	0.085
18	2.546	2.334	2.585	2.587	1.244	1.154	1.304	1.382	0.898	0.924	0.911	0.978	0.261	0.343	0.336	0.351
19	2.899	2.828	2.653	2.628	1.255	1.205	1.15	1.106	0.766	0.797	0.854	0.852	0.155	0.244	0.244	0.277
20	3.207	3.064	2.953	2.903	1.424	1.409	1.285	1.24	0.755	0.895	0.918	0.935	0.268	0.291	0.355	0.39

Assay no	216				217				anti-Pgp3 pAb				anti-GST mAb			
1	0.142	0.061	0.096	0.085	0.044	0.04	0.031	0.023	2.863	2.72	3.163	3.313	1.666	1.835	1.609	1.321
2	0.09	0.067	0.101	0.084	0.09	0.004	0.053	0.057	2.709	2.755	2.825	2.879	1.295	1.334	1.3	1.327
3	0.063	0.057	0.101	0.092	0.024	0.004	0.047	0.036	3.056	2.982	3.303	3.161	1.516	1.651	1.837	1.803
4	0.1	0.077	0.096	0.093	0.008	0.007	0.021	0.015	3.002	3.105	3.037	3.254	1.257	1.279	1.259	1.392
5	0.155	0.127	0.126	0.099	0.007	0.005	-0.02	-0.04	2.96	3.188	2.927	3.063	1.285	1.235	1.388	1.422
6	0.024	-0.01	0.117	0.089	0.016	-0	0.022	-0	2.845	2.822	3.399	3.428	1.738	1.777	1.499	1.606
7	0.161	0.095	0.12	0.11	-0.03	-0.04	-0.06	-0.06	3.039	2.952	3.139	3.292	1.947	1.97	1.738	1.809
8	0.128	0.084	0.169	0.14	0.016	0.006	0.023	-0.01	2.627	2.736	3.251	3.308	1.634	1.804	1.962	2.002
9	0.117	0.079	0.101	0.142	0.173	0.089	0.047	0.052	2.232	2.516	2.998	3.103	1.403	1.422	1.517	1.484
10	0.112	0.085	0.143	0.11	0.033	0.025	0.037	0.033	3.01	3.01	3.165	3.093	1.714	1.909	1.477	1.638
11	0.054	0.041	0.052	0.043	0.052	0.024	0.07	0.064	3.209	3.109	3.185	2.975	1.809	1.497	1.945	0.973
12	0.172	0.137	0.21	0.093	0.068	0.031	0.022	0.087	2.475	2.657	2.753	2.68	1.353	1.381	1.305	1.499
13	0.105	0.071	0.086	0.076	0.071	0.02	0.053	0.032	2.75	3.357	3.277	3.143	1.326	1.383	1.364	1.421
14	0.14	0.12	0.184	0.155	0.05	0.044	0.032	0.073	3.024	3.016	2.959	2.806	1.364	1.386	1.319	1.411
15	0.076	0.061	0.065	0.051	0.048	0.019	0.038	0.037	2.876	2.814	2.877	2.901	1.28	1.351	1.171	1.343
16	0.138	0.101	0.126	0.1	0.033	0.027	0.025	0.008	3.104	2.995	2.916	2.972	1.487	1.576	1.518	1.573
17	0.164	0.135	0.17	0.155	0.1	0.04	0.033	0.035	2.994	2.892	3.132	2.966	1.517	1.478	1.469	1.588
18	0.134	0.11	0.17	0.135	0.096	0.095	0.097	0.065	2.724	2.978	2.926	2.765	1.901	1.879	1.856	1.968
19	0.071	0.104	0.175	0.112	0.123	0.102	0.128	0.1	3.456	3.456	3.336	3.336	1.683	1.68	1.471	1.848
20	0.161	0.091	0.142	0.127	0.152	0.137	0.096	0.101	3.457	3.457	3.326	2.625	1.598	1.672	1.426	1.617

APPENDIX V: LEVEY JENNINGS CHARTS AND CONTROL LIMITS

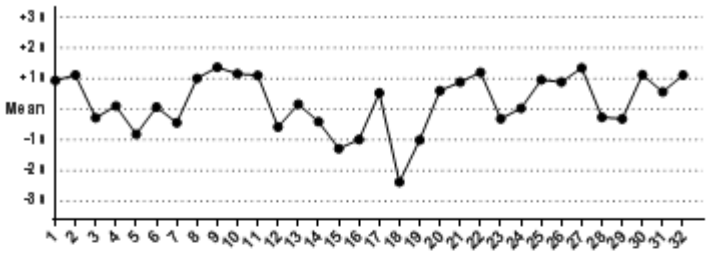
MULTIRULE QUALITY CONTROL WESTGARD RULE CONTROL LIMITS AND LEVEY-JENNINGS CHARTS FROM SEROPREVALENCE STUDIES (CHAPTER SIX)

Upper and lower control limits of serum and positive antibody controls used for the GST-PGP3 SW2 indirect ELISA. Control limit OD values were calculated using mean background-corrected duplicate OD values obtained from two plates across twenty independent assays (5.3.3). Using the GST-PGP3 indirect ELISA, sera from patients were assayed at 1:100 and mouse monoclonal anti-GST and rabbit polyclonal anti-PGP3 antibodies were assayed at 1:10,000. Experiments were conducted using GST-PGP3 SW2 batches no 4 and 5.

Serum No. /Control	-3_S	-2_S	-1_S	Mean	$+1_S$	$+2_S$	$+3_S$
204	2.405	2.579	2.753	**2.927**	3.101	3.275	3.449
205	0.889	1.045	1.201	**1.357**	1.513	1.669	1.825
234	0.62	0.721	0.822	**0.923**	1.024	1.125	1.226
215	0.028	0.112	0.196	**0.28**	0.364	0.448	0.532
216	0.015	0.046	0.077	**0.108**	0.139	0.169	0.201
217	-0.078	-0.038	0.002	**0.042**	0.082	0.122	0.162
Anti-Pgp3	2.483	2.659	2.835	**3.011**	3.187	3.363	3.539
Anti-GST	0.962	1.157	1.352	**1.547**	1.742	1.937	2.132

Levey-Jennings charts of serum controls from GST-PGP3 SW2 assays (Westgard Rules). Graph shows the mean duplicate OD_{450} values obtained from six control sera from adults (assayed at 1:100) and anti-GST and anti-PGP3 antibodies (1:10,000) assayed against GST-PGP3 SW2 antigen in duplicate over thirty-two independent ELISAs. Data was calculated using GraphPad Prism version 6.0.

204



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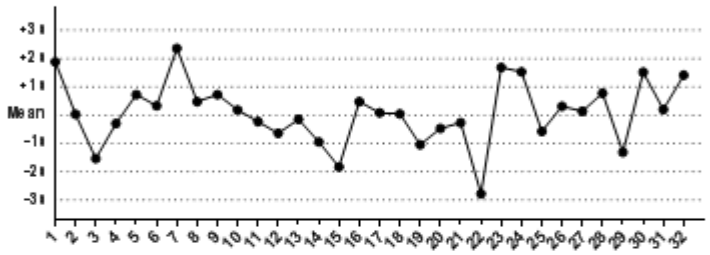

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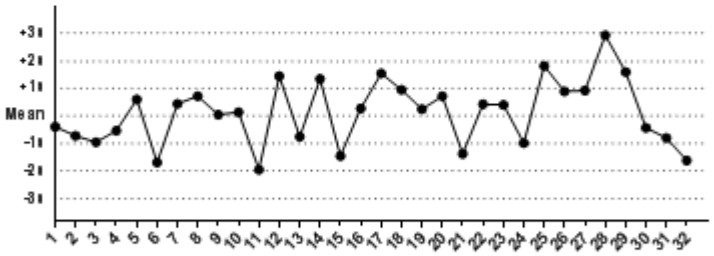
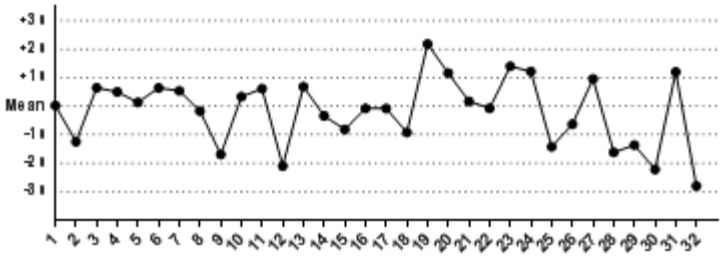


anti-Pgp3



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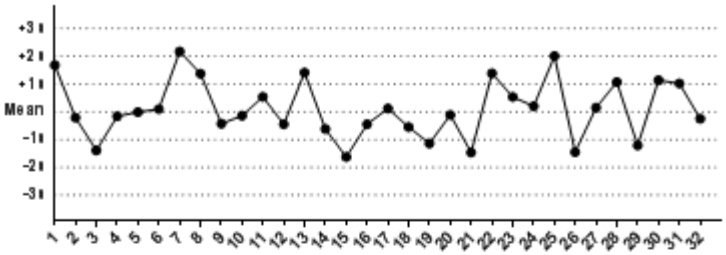

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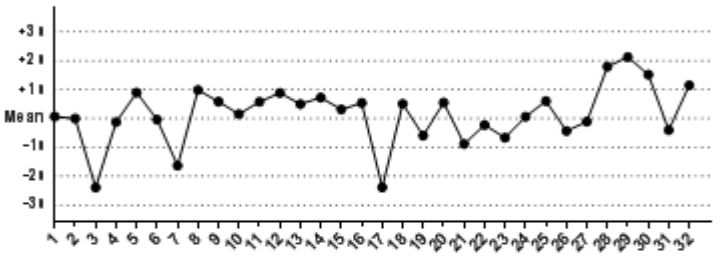

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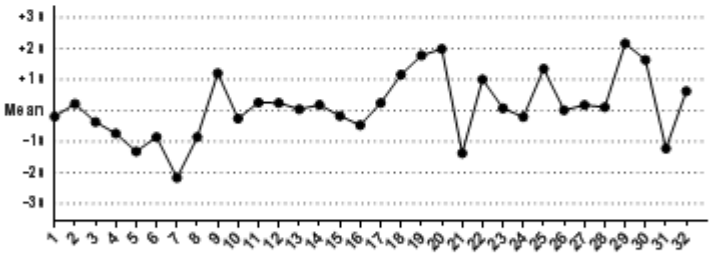
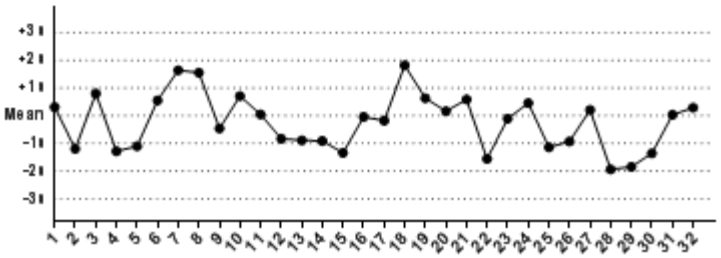


anti-GST



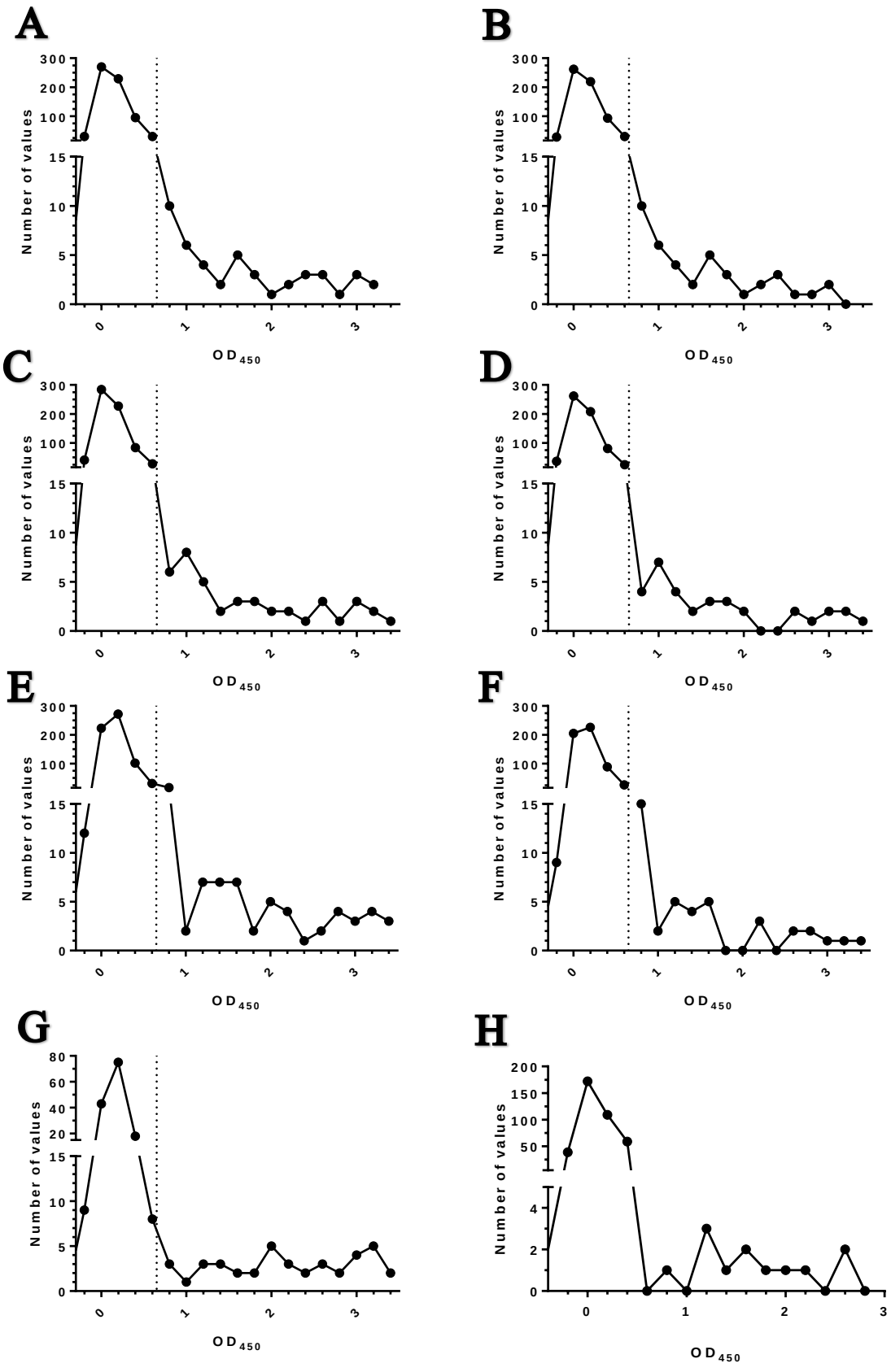
APPENDIX VI: DESCRIPTIVE STATISTICS AND OD FREQUENCY DISTRIBUTION OF SEROPREVALENCE DATA

DESCRIPTIVE STATISTICS OF SEROPREVALENCE DATA AND GRAPHS SHOWING FREQUENCY DISTRIBUTION OF OD VALUES OBTAINED FROM SERA COLLECTED FROM 13-15-YEAR-OLDS ASSAYED AGAINST GST-PGP3 SW2 IN THE ELISA (CHAPTER SIX)

Descriptive statistics of OD_{450} values from patient sera assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST by indirect ELISA. GraphPad Prism 6.0 was used to calculate the median, mean and standard deviations of the background-corrected OD_{450} values obtained from 2119 serum samples collected from 13-15-year-old patients assayed against GST-PGP3 derived from *C. trachomatis* pSW2 in the indirect ELISA.

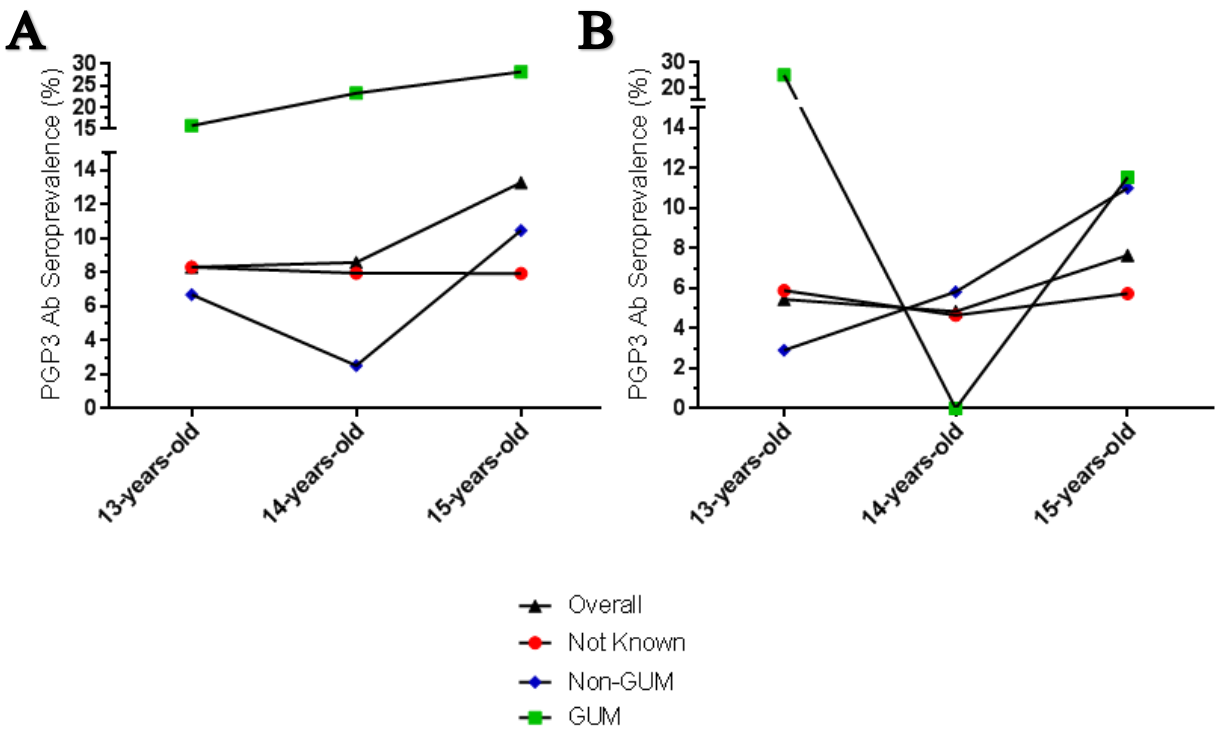
	13-year-olds		14-year-olds		15-year-olds		GUM samples only	Samples assayed against GST
	All samples	Excluding GUM	All samples	Excluding GUM	All samples	Excluding GUM		
Median	0.130	0.129	0.118	0.115	0.170	0.157	0.208	0.080
Mean	0.245	0.230	0.225	0.215	0.324	0.261	0.584	0.149
SD	0.451	0.395	0.456	0.424	0.550	0.417	0.908	0.337

Frequency distributions of OD_{450} values from patient sera assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 and GST by indirect ELISA. GraphPad Prism 6.0 was used to calculate the frequency distributions of the background-corrected OD_{450} values obtained from 2119 serum samples collected from 13-15-year-old patients assayed against GST-PGP3 derived from *C. trachomatis* pSW2 in the indirect ELISA. Graphs **[A]-[G]** show the frequency distribution of OD_{450} values of sera assayed against GST-PGP3 SW2. The dotted lines on **[A]-[G]** represent the OD_{450} 0.652 cut-off point for females (cut-off for males = OD_{450} 0.665). **[A]** 13-year-olds: all serum sample groups (GUM/non-GUM/unknown sources); **[B]** 13-year-olds: GUM samples excluded (non-GUM/NK included only); **[C]** 14-year-olds: all serum groups; **[D]** 14-year-olds: GUM samples excluded. **[E]** 15-year-olds: all serum groups; **[F]** 15-year-olds: GUM samples excluded; **[G]** 13-15-year-olds combined: GUM serum samples only; **[H]** Serum samples assayed against GST.


A
B
C
D
E
F
G
H
Number of values
OD450

APPENDIX VII: EXTRA FIGURES AND TABLES FROM CHAPTER SIX

Seroprevalence of anti-PGP3 antibodies in 13-15-year-olds in England by age group and source of serum collection. Graphs show the prevalence (%) of anti-PGP3 antibodies found in 2107 serum samples collected from males and females assayed against recombinant GST-PGP3 derived from *C. trachomatis* pSW2 in an indirect ELISA. **[A]** Serum samples from female patients; **[B]** Serum samples from male patients.



APPENDIX VIII: GST-PGP3 SW2 ELISA METHODS PAPER

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Research paper

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 ($p \leq 0.0001$).
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.

1. 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

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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 (Appendix S1). 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.

2. Materials and methods

2.1. 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 *Xho*I and *Bam*HI (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.

2.2. 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 h 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 h 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 h then at 4 °C for 16 h. Pooled fractions were dialysed against PBS overnight at 4 °C using CelluSep T1 cellulose membrane (MWCO: 3500) (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.

2.3. 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 min at 100 °C and analysed by SDS polyacrylamide gel electrophoresis on 12% gels.

2.4. 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 >5 freeze-thaw cycles.

2.5. 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

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us to separate results based on sex and diagnosed *C. trachomatis* infection.

2.6. 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 2 h 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 h 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

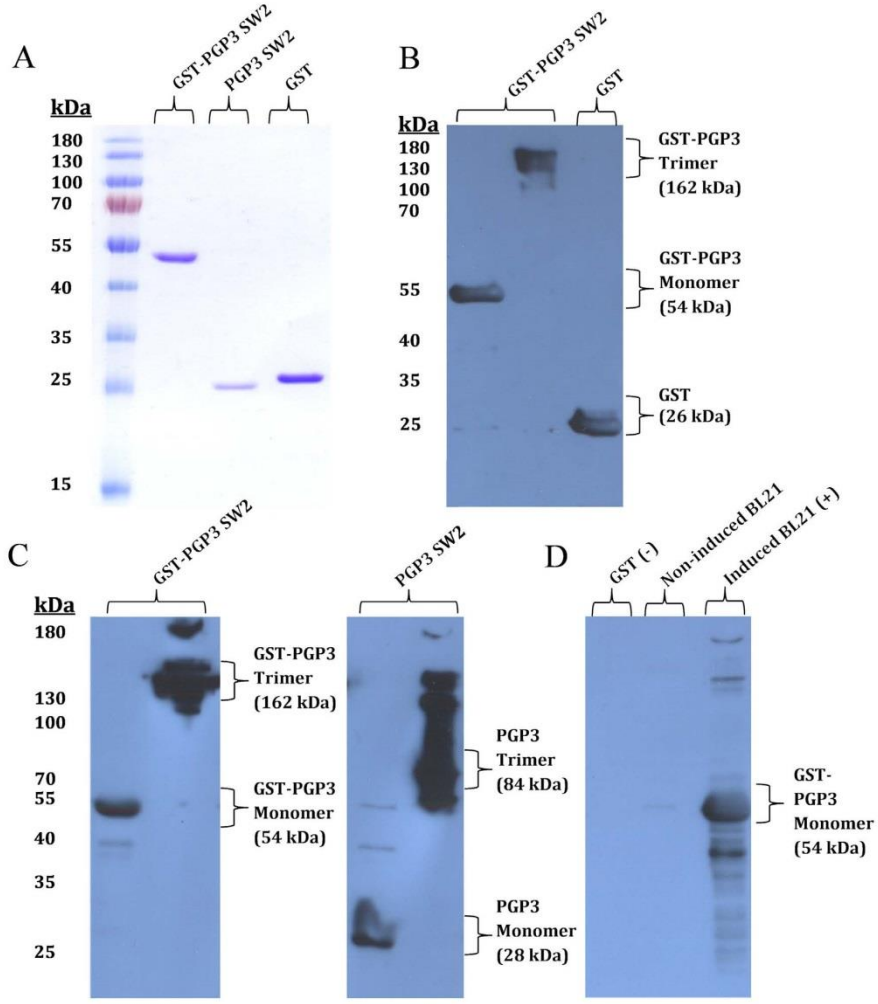

Fig. 1. Analysis of recombinant PGP3 expression and trimerisation by polyacrylamide gel electrophoresis. Recombinant PGP3 derived from *C. trachomatis* pSW2 (serovar E) expressed as a fusion protein with an N-terminal glutathione s-transferase tag was purified from an *E. coli* expression system in which 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 min, 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 h. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: Winstanley, C.E., et al., Development and evaluation of an enzyme-linked immunosorbent assay for the detection of antibodies to a common urogenital derivati..., J. Immunol. Methods (2017), http://dx.doi.org/10.1016/j.jim.2017.03.002

(100 μl/well) for 1 h 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 100 μl/well of ready-made 3,3′,5,5′-tetramethylbenzidine (TMB) solution (eBioscience) for 10 min. The reaction was stopped with 50 μl/well 2 M 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.

2.7. 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 min, 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 h at room temperature (RT), membranes were washed three times and incubated with human (1:200) primary antibodies for 1 h at RT. Membranes were washed and incubated with secondary antibodies diluted 1:2000 for 1 h 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.

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

3. 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: 865,439) and F (pSW4; EMBL: 865,441) share a 100% amino acid identity.

3.1. Configuration, expression and purification of recombinant PGP3

Recombinant GST-PGP3 can be easily and efficiently expressed, purified and dialysed against PBS in <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 Fig. 1A. Monomers and trimers of GST-PGP3 and PGP3 were observed using Western blot (Fig. 1B and C). 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* (Fig. 1D). No reaction to GST or any non-specific products were observed.

3.2. 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

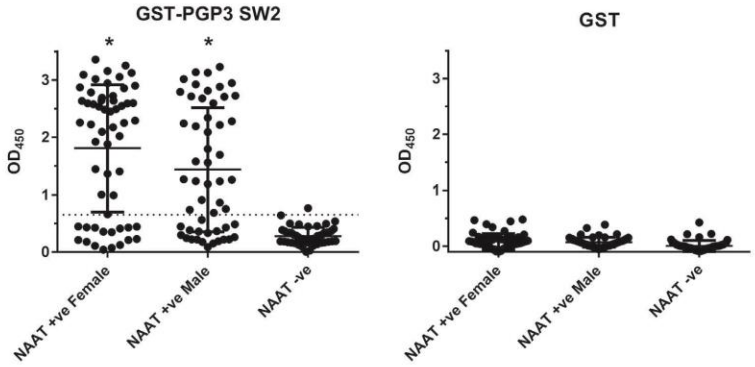

Fig. 2. Antibody responses to GST-PGP3 by indirect ELISA using patient sera. Graphs show background-corrected mean OD_{450} values of sera collected from patients who had been tested for *C. trachomatis* by NAAT. Sera were assayed in duplicate at 1:100 against N-terminal glutathione s-transferase-tagged 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 ($p \leq 0.0001$) 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].

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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 $p \leq 0.05$.

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)

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 (Fig. 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).

3.3. 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 (Fig. 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: $p \leq 0.0001$). All patient sera returned a background-corrected OD_{450} value against GST below 0.481 (Fig. 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 (Fig. 3 and Appendix S2), indicating possible past exposure to *C. trachomatis* as these patients are likely to have been sexually active.

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. (2009) and 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. (2009) and 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.

3.4. 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. (2001b) also

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

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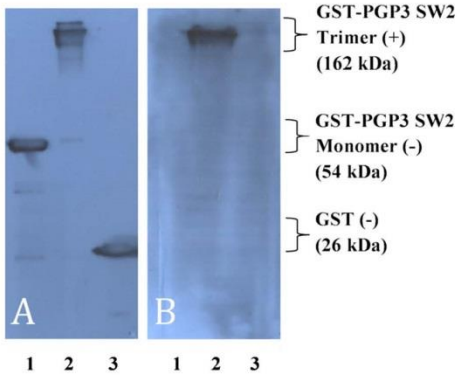

Fig. 3. Human antibody recognition of PGP3 in a western blot using sera from a patient who tested negative for *C. trachomatis* by NAAT. GST-PGP3 derived from *C. trachomatis* pSW2 (serovar E) was treated (lane 1: 2% SDS, 1.25% β-mercaptoethanol, heat boiled for 5 min) or untreated (lane 2), analysed 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.

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).

3.5. 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 and 12.6% for inter-assay variation and between 2.64 and 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.

Taken together these results show we have developed a robust, reliable and accurately reproducible serological assay using PGP3 in the

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**

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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.

4. 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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APPENDIX IX: SEROPREVALENCE DATA

RAW AND BACKGROUND-CORRECTED DATA OBTAINED FROM 13-15-YEAR-OLD SEROPREVALENCE STUDIES (CHAPTER SIX).

Serum samples from 13-14-year-olds (serum set 'b') were assayed in July-August 2016 Serum samples from 15-year-olds (serum set 'a') were assayed in August 2015. Serum set 'a' samples were assayed using GST-PGP3 SW2 batch no 5 and serum set 'b' samples were assayed using batch no 6. Both serum sets were assayed using GST (4T1) batch no 2.

This data can be found saved to the CD attached to the back of this thesis.

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