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Chlamydia trachomatis serology as a means of monitoring intervention activities
to eliminate trachoma as a public health problem

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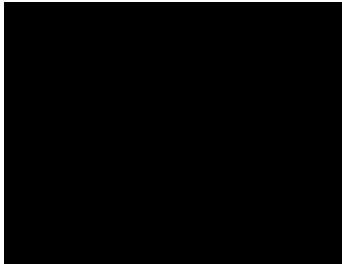
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Declaration

I, Stephanie Jasmine Migchelsen, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.



Signature Date 19 September 2018

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Preface

This thesis is presented as a 'Research Paper Style Thesis' in accordance with submission guidance provided by the London School of Hygiene and Tropical Medicine. Four of the chapters comprise papers that have been published or are prepared for submission to peer-reviewed journals. These are highlighted in italics in the Table of Contents. In view of the differing requirements of the journals in which the work has been published there is by necessity some repetition of material and variation in the formatting of these chapters. Publication details and acknowledgement of co- author contributions are included on the individual cover sheets for each paper. The remainder of the thesis is comprised of 'linking material' and includes an introduction to the overall research project.

All material within this thesis was written by Stephanie Migchelsen.

Abstract

Trachoma causes blindness as a result of repeated ocular infection with *Chlamydia trachomatis* (Ct). International efforts are focused on eliminating trachoma as a public health problem by 2020; the World Health Organisation's (WHO) and the Global Alliance for the Elimination of Trachoma by 2020 (GET2020) (1) recommend the SAFE strategy (Surgery to treat trichiasis, Antibiotics to treat infection with Ct, Facial cleanliness and Environmental change to reduce transmission) (2). Current implementation guidelines for the A, F and E aspects of the strategy are based on the prevalence of trachomatous inflammation-follicular (TF) in children aged 1-9 years. Antibiotics are administered as azithromycin mass drug administration (MDA) to all residents of districts wherein the prevalence of TF in 1-9 year old children is greater than 10% (3).

As we approach the global elimination of trachoma, the prevalence of TF in children will decline towards the elimination threshold of 5%, and with it, the positive predictive value of TF. This may result in inappropriate, continued administration of antibiotics, which is a mis-use of valuable financial and person resources, as well as raising concerns about antimicrobial stewardship.

Once the prevalence of TF in children aged 1-9 years is below 5%, re-emergence must be monitored. The WHO recommends a 'pre-validation' survey to determine if re-emergence has occurred (4). The 2014 Technical Consultation on Trachoma Surveillance (4) recommended exploring the district-level prevalence of TF, the district-level prevalence of conjunctival infection with Ct and the district-level prevalence of antibodies against Ct, to determine an appropriate measure, or combination of measures, for deciding when to stop MDA.

Numerous methods exist for detecting antibodies against Ct antigens (5–9). Specimens may be collected by venepuncture or from fingerprick, stored as whole blood, serum or dried blood spots on filter paper, and assayed using enzyme-linked immunosorbent assays (ELISA), lateral flow assays (LFA), or multiplex bead assay (MBA); of these, MBA allows for the testing for antibodies against numerous antigens from endemic infections, but the required instrumentation and reagents are more cost-intensive. Historically, serology for Ct fell out of favour due to the high cross-reaction with antigens from *C pneumoniae*, however, with the advent of new proteomic tools and assays with greater reported sensitivity and specificity, serology has re-emerged as a potential tool for monitoring the prevalence of antibody-inducing chlamydial infection (4,10,11).

Both ELISA and MBA assays present output as numerical data, which must be translated to a population seroprevalence value. Therefore, a method must be determined for dichotomising numerical data and setting a threshold between seropositive and seronegative samples. Receiver

Operating Characteristic (ROC) curves have previously been used, but these rely on appropriate reference standards. Alternate methods that rely solely on the data generated within a study, such as finite mixture modelling, may be more appropriate (12–15).

Once seropositivity has been estimated, it is of interest to detect changes in the age-specific seroprevalence of a population. Catalytic models have previously been used to monitor changes in the prevalence of malaria (13,16–22) and this methodology can be applied to detect changes in transmission as a proxy for the force of infection (FOI) as well as to estimate seroconversion rates, and to a lesser extent, seroreversion rates (13,19).

The simplicity of dichotomous seroprevalence estimates may provide lower resolution information than the quantitative antibody levels. Recent work uses quantitative antibody levels to measure changes in transmission (23). Ensemble machine learning can be used to produce characteristic age-specific antibody curves that may reveal changes in population mean antibody levels that would otherwise be masked because changes occurred above or below the seropositivity threshold.

There remain several areas to be elucidated. While antigens used in modern serological studies are considered specific for Ct, there is yet no way to distinguish between antibodies due to ocular infection or genital infection, making serological studies in anyone over the age of sexual debut a challenge. There is no standard reference for antibody levels, making a comparison between different studies challenging. The two most commonly used assays produce different output data: ELISAs measures optical density- the amount of light absorbed by the specimen- while MBAs detect fluorescence in an assayed specimen. An internal standard would allow for comparison between the two assays.

This PhD research addresses several key questions about the use of serology and Ct-specific antibodies for monitoring the prevalence of trachoma. As more countries progress to eliminating trachoma as a public health problem by 2020, efforts will need to be increased to monitor and evaluate elimination efforts and to prevent re-emergence of the disease. Serological techniques may be ideal for such activities.

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Glossary of abbreviations and terms

95% CI	95% confidence interval
active trachoma	The condition of having either trachomatis inflammation- follicular (TF) and/or trachomatous inflammation- intense; see also ‘scarring trachoma’
<i>alkalo</i>	Village chief in The Gambia
aOR	Adjusted odds ratio
Catalytic model	A means of modelling to describe mathematically the rate of change of a variable as a function of time.
CDC	Centers for Disease Control and Prevention
CF	complement fixation
cHsp	Chlamydial heat shock protein; see also ‘heat shock protein’
CO	Corneal opacity; corneal scarring that blurs the pupil margin
CoV	Coefficient of variation; ratio of the standard deviation within a group of samples to the mean of the samples
Cohen’s kappa	A statistical measure of agreement between categorical items.
Control	Reduction of disease incidence, prevalence, morbidity, and/or mortality to a locally acceptable level as a result of deliberate efforts (WHO, 2015)
Cp	<i>Chlamydia pneumoniae</i>
<i>C. psittaci</i>	Species within the genus of <i>Chlamydia</i> , causes avian chlamydiosis and respiratory psittacosis in humans.
CRD	Clinical Research Department
Ct	<i>Chlamydia trachomatis</i>
CT694	Protein secreted by Ct involved in pathogenesis and the secretory process
DBS	Dried blood spot
ddPCR	droplet digital PCR
DNA	Deoxyribonucleic acid
EA	Enumeration area- census area, as defined by the Gambian government
EB	elementary body- nonreplicating infectious particles that are released when infected host cells rupture
Elimination as a public health problem	Achievement of measurable global targets set by WHO in relation to a specific disease; unless otherwise specified, in this work the term ‘elimination’ refers to elimination as a public health problem
Elimination of transmission	Reduction to zero of the incidence of infection caused by a specific pathogen in a defined geographical area, with minimal risk of reintroduction, as a result of deliberate efforts (WHO, 2015)
ELISA	Enzyme-linked immunosorbent assay
EM	expectation-maximisation algorithm- iterative method to find maximum likelihood estimates of parameters in a statistical model

ESPEN	Expanded Special Project for the Elimination of Neglected Tropical Diseases
FoI	Force of Infection; the rate at which susceptible individuals acquire an infectious disease
FMM	finite mixture model- probabilistic model for representing the presence of two or more subpopulations within the overall population
GTMP	Global Trachoma Mapping Project
GUM	Genito-urinary medicine; see also STI
hotspot	Defined as a community in a district with a prevalence of TF higher than 10% when all other surveyed communities are below 10%. Note that this is not an official WHO definition but has been used in a country-specific example in Lao PDR (Southisombath et al., 2016).
HRP	Horseradish peroxidase
Hsp	heat shock protein; protein produced by cells in response to stressful conditions
hyperendemic	A population in which the prevalence of active trachoma (TF and/or TI) in children aged 1–9 years old is >20% (Wright & Taylor, 2005)
hypoendemic	A population in which the prevalence of active trachoma in children aged 1–9 years old is <10% (Wright & Taylor, 2005)
hysterosalpingogram	radiologic procedure to investigate the fallopian tubes
Ig	Immunoglobulin; summarise types written about
IIF	indirect immunofluorescence
ITD	Infectious and Tropical Diseases
ITI	International Trachoma Initiative
laparoscopy	Keyhole surgery to examine and/or treat fallopian tubes, ovaries or uterus
LFA	lateral flow assay
LGV	lymphogranuloma venereum
LRR	Lower River Region
LSHTM	London School of Hygiene and Tropical Medicine
MBA	multiplex bead assay
MDA	mass drug administration of antibiotics
Mesoendemic	A population in which the prevalence of active trachoma
MFI-bkgd	Median fluorescent intensity minus background, unit of measurement for MBA results
MIF	micro-immunofluorescence
MOMP	39 kilodalton immunodominant major outer membrane protein
MRC	Medical Research Council
NAAT	Nucleic acid amplification test
NG	<i>Neisseria gonorrhoeae</i>
NTD	neglected tropical disease
<i>ompA</i>	gene that encodes the major outer membrane protein (MOMP)
OD ₄₅₀	Optical density of an ELISA measured at 450nm

OR	Odds ratio
ORF	Open reading frame; part of an organism's genetic code that can be translated to produce a protein
PBS	Phosphate-buffered saline
PBSTw	PBS with 0.3% v/v Tween 20
PCR	polymerase chain reaction; technique to make many copies of a specific DNA sequence
PCT	Preventive chemotherapy and transmission control focus on diseases for which a strategy exists as well as on tools and the availability of safe and effective drugs that make it feasible to implement large-scale preventive chemotherapy.
peri-elimination	A region or country in which trachoma is believed to have been eliminated as a public health problem but has not yet been validated. It is expected that the prevalence of TF in children aged 1-9 years would be less than 5%.
perihepatitis	Inflammation of the coating of the liver, often caused by inflammation of the upper genital tract in women, commonly known as pelvic inflammatory disease (PID)
PID	Pelvic inflammatory disease
Pgp3	plasmid gene product 3
PPV	positive predictive value
R ₀	basic reproduction number; the average number of infections that occur from an index case in a fully susceptible population
RCM	Reverse catalytic model
ROC curve	Receiver Operating Characteristic curve; a graphical plot to illustrate the diagnostic ability of a dichotomous classification system as its threshold is varied.
SAFE	surgery, antibiotics, facial cleanliness, environmental improvements
scarring trachoma	The condition of having scarring in the tarsal conjunctiva (TS) and/or trichomatous trichiasis (TT) and/or corneal opacity (CO) as a result of ocular infection with Ct; see also 'active trachoma'
SCR	seroconversion rate (λ); the yearly average rate by which seronegative (never infected) individuals become exposed to a disease and produce antibodies against that specific disease
SD	standard deviation; a measure to quantify the amount of variation within a set of values
seroconversion	The process by which specific antibody develops and becomes detectable in the blood by a serological assay; antibody levels fall above a threshold for positivity
seroreversion	The point at which antibody is produced at insignificant amounts and is too low to be detected; antibody levels fall below a threshold for positivity

serovar	A distinct variation of subspecies within Ct classified based on cell surface antigens allowing for epidemiological classification. Ct serovars tend to show tropism for ocular or genital regions
SRR	seroreversion rate (δ); the annual mean rate by which seropositive individuals revert to a seronegative status in the absence of re-infection
STI	sexually transmitted infection
TF	trachomatous inflammation–follicular; the presence of five or more follicles in the upper tarsal conjunctiva
TFI	tubal factor infertility
TI	trachomatous inflammation–intense; the pronounced inflammation and thickening of the upper tarsal conjunctiva to obscures the normal deep tarsal vessels
TMB	tetra-methylbenzidine
TRA	Trachoma rapid assessment-a rapid survey based on a convenience sample of communities where trachoma is likely to be present
TS	Trachomatous scarring; the presence of scarring in the tarsal conjunctiva
TT	Trachomatous trichiasis; at least one eyelash rubbing the surface of the eyeball
TV	<i>Trichomonas vaginalis</i>
URR	Upper River Region
validation	The process by which elimination of a disease as a public health problem is documented (WHO, 2015)
VIP	Visual Inflection Point-an estimation of the point of inflection of a curve based on visual, human examination
WHO	World Health Organization
WIF	whole cell inclusion immunofluorescence
Youden’s J-index	A statistic to capture the performance of a dichotomous diagnostic test. Measured $J = \text{sensitivity} + \text{specificity} - 1$

List of contributors to the research presented in this thesis

Name	Position/Institution	Contribution
David Mabey	Professor of Communicable Disease, CRD, ITD, LSHTM	PhD Supervisor
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Nuno Sepulveda	Assistant Professor of Biostatistics and Statistical Genetics, ITD, LSHTM	Essential support with seroconversion scripts
Diana Martin	Research Epidemiologist, CDC	Essential support with multiplex bead assay
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Thesis Outline

This thesis is composed of explanatory materials, data chapters based on published and submitted manuscripts and linking material to create a logical flow. Chapters highlighted in italics consist of papers that have been published or submitted for publication. The other chapters are linking material prepared for this thesis.

Chapter 1 is an overview of the components of my PhD research and the components of this project. It outlines the scientific rationale for my research.

Chapter 2 provides the history of serology for the investigation of *Chlamydia trachomatis* (Ct) infection, an overview of assays, antigens and immunoglobulins, before reviewing the studies performed to-date, looking at both those specific to ocular and genital Ct infection.

Chapter 3 details the development of the Pgp3-specific ELISA used to analyse samples from The Gambia, Laos, Uganda and Kiribati. The ELISA has also been used by other researchers to analyse sample from serological studies in Ghana, Malawi and the Solomon Islands.

Chapter 4 presents serological data from The Gambia, Uganda and Laos and methods in which thresholds for serological data can be set to separate seropositive samples from seronegative samples. Results suggest that methods relying only on the samples themselves, rather than external controls, produce more consistent results. This chapter also details the Pgp3-specific ELISA used to generate serological data.

Chapter 5 uses a reversible catalytic model to estimate seroconversion and seroreversion rates in The Gambia. Evidence suggests an abrupt change in transmission occurred approximately 20 years prior to samples being collected, in addition to a more gradual decline in disease and infection as access to water, healthcare and education increased from 1986 to the current day.

Chapter 6 examines age-standardised antibody acquisition models as an alternative method to dichotomised seropositive results. Results are compiled from seven serological studies from endemic and previously-endemic countries across a range of ages. Age-dependent antibodies level provide proof of changes between pre- and post-MDA in a population, the ability to distinguish between populations with on-going transmission and ones in which trachoma has been eliminated and, at population-level, can approximate when Ct infection is acquired, whether ocular or genital infections.

Chapter 7 provides the final discussion and conclusion to the research presented in this thesis.

Chapter 1 Overview of PhD objectives

1.1 Project Summary

Trachoma is a form of chronic conjunctivitis caused by ocular infection with the obligate intracellular bacterium *Chlamydia trachomatis* (Ct). It is the world's most common form of infectious and preventable blindness and affects people predominantly in the most remote and poorest areas of the world. The World Health Organization (WHO) estimates that 190 million people live in trachoma-endemic regions; 21 million people have active trachoma, and 7.3 million people need surgery for trachomatous trichiasis. Trachoma has led to visual impairment in 1.9 million people of whom 1.2 million are blind [1].

Diagnosis of trachoma is based on the presence of clinical signs. Active trachoma is characterised by trachomatous inflammation. Trachomatous inflammation- follicular (TF) is defined as the presence of five or more follicles, each at least 0.5mm in diameter in the central part of the upper tarsal conjunctiva [2]. Trachomatous inflammation-intense (TI) indicates inflammation obscuring at least half the tarsal blood vessels [2]. Repeated infection over the course of a lifetime can lead to harmful sequelae known as scarring trachoma: trachomatous scarring (TS) in which scars are easily visible as white lines or bands in the tarsal conjunctiva; trachomatous trichiasis (TT) with at least one eyelash touching the surface of the eyeball; and corneal opacity (CO) when the repeated scratching of the eyeball leads to corneal scarring blurring the pupil margin and reducing visual acuity, leading ultimately to blindness [2].

The WHO Roadmap proposed to eliminate trachoma as a public health problem by 2020 [3]. Elimination as a public health problem is related to both infection *and* disease, defined by measurable targets set by the WHO; continued actions are required to maintain targets and advance the interruption of transmission. Validation is the process of documenting the elimination of a disease as a public health problem [4]. The operational thresholds for the elimination of trachoma as a public health problem are defined as: (i) a prevalence of trachomatous trichiasis (TT) "unknown to the health system" of < 1 case per 1000 total population; and (ii) a prevalence of trachomatous inflammation-follicular (TF) in children aged 1–9 years of < 5%, in each formerly endemic district [5].

Elimination of trachoma as a public health problem is based on the SAFE strategy: **S**urgery for trichiasis, **A**ntibiotics to reduce infection, **F**acial cleanliness to reduce transmission and **E**nvironmental improvement. The SAFE strategy comprises both preventive chemotherapy and transmission control (PCT) as well as hygiene and environmental management. Antibiotics in the form of azithromycin (and tetracycline eye ointment when contra-indicated) are distributed to all

members of communities wherein the prevalence of TF is greater than 10% in children aged 1-9 years [6]. Substantial international effort has gone into the elimination of trachoma as a public health problem, with the Global Trachoma Mapping Project collecting data from 2.6 million people across 29 countries, to determine the prevalence of trachoma in these countries and their requirement for the elements of the SAFE strategy [7].

With the leadership of the WHO, the global effort to eliminate trachoma as a public health problem has seen significant success, with several countries declared as having met the elimination targets: as of October 2018, Cambodia, China, Gambia, Ghana, Islamic Republic of Iran, Iraq, Lao People's Democratic Republic, Mexico, Morocco, Myanmar, Nepal and Oman had reported achieving elimination goals [1,8,9].

As efforts to eliminate blinding trachoma as a public health problem are increased to meet the 2020 deadline [10], experts have agreed to explore the correlation between clinical signs, infection and antibodies, and how these can be used to advise programmatic decisions regarding the implementation, continuation and eventual cessation of MDA of antibiotics [11]. Studies monitoring elimination efforts have previously measured the prevalence of disease [12–17], however there are concerns that surveys based on clinical signs in post-MDA communities may lead to ongoing interventions that are unnecessary and inappropriate: active trachoma (the presence of TF and/or TI) often persists after infection has cleared, these clinical signs may be overcalled (due to the challenge of standardised training) [18] or that follicular diseases may have different aetiology [19]. In post-MDA communities, the positive predictive value (PPV) of clinical signs is diminished [20]. Finally, there is no biological significance to the 5% TF prevalence as set by the WHO for either stopping MDA or predicting the recrudescence of active trachoma [21].

Surveillance efforts have also used PCR to estimate the prevalence of ocular Ct infection [22–26], however monitoring active ocular Ct infection is expensive with one test for infection estimated to cost \$8-16 (US Dollars) per test [21] and no thresholds for the prevalence of ocular Ct infection have been suggested by the WHO.

In 2014, the Technical Consultation on Trachoma Surveillance made recommendations to explore alternative indicators on which to base the decision to stop MDA; these include the district-level prevalence of antibodies to *C. trachomatis*-derived antigens [11]. A change in the prevalence of seropositivity in a community may demonstrate when the level of Ct circulating in a community has reached a level low enough to prevent recrudescence of infection and disease.

Serological assays for Ct-specific antibodies have existed for many years and the historical context is provided in detail in Chapter 2 of this thesis. In brief, early assays were non-specific and often cross-reacted with antibodies against *C. pneumoniae* (Cp) [27,28]. Additionally, concerns about the sensitivity and specificity of previous assays have been raised [29–31]. Advances in protein expression and purification techniques and the development of multiplex technology has led to the development improved assays [32–34], although in resource-poor settings, a less technologically-dependent assay may be preferable.

The research conducted as part of this thesis aims to explore serology as a means of monitoring intervention activities. This thesis first reviews the historical use of serology for detection antibodies against Ct infection and summarises the use of Ct-specific serological assays at a population level to measure the prevalence of antibodies resulting from ocular and genital infections. The development of a Pgp3-specific ELISA is presented in Chapter 3 along with methods to set seropositivity thresholds in Chapter 4. Using age-standardised seroprevalence, the seroconversion and seroreversion rates were modelled under different epidemiological settings in Chapter 5. Finally, in Chapter 6, antibody acquisition models were used to compare datasets from countries where trachoma has been eliminated as a public health problem to those where transmission is on-going and further, where the prevalence of genital Ct infection is likely contributing to the prevalence of Ct-specific antibodies in the population. The final chapter summarises the work completed for this thesis and addresses the epidemiological implications of this research and suggests future research.

1.2 Aims and Objectives

1.2.1 Aim

The aim of this research was to measure antibodies against Ct, using a Pgp3-specific ELISA, and use the age-specific seroprevalence as an alternative means of measuring the prevalence of ocular Ct infection.

1.2.2 Specific Objectives

1. Collect demographic and clinical information and fingerprick blood samples to investigate the epidemiology of trachoma and the age profiles of antibodies against Pgp3 in Lower and Upper River Regions of The Gambia
2. Develop a Pgp3-specific ELISA to measure antibodies against Ct using fingerprick dried blood spots (DBS)

3. Determine appropriate cut-off thresholds to distinguish between seropositive and seronegative individuals in populations from The Gambia, Lao PDR and Uganda.
4. Use seroprevalence data to estimate changes in transmission in The Gambia and estimate seroconversion and seroreversion rates.
5. Use antibody level data as an alternative to seroprevalence data to measure changes in Ct transmission

1.3 Hypotheses

1. Antibodies against Pgp3 can be measured using a sensitive and specific ELISA.
2. Internally-calibrated thresholding methods are an appropriate means of determining seropositivity thresholds.
3. Serology can be used to infer changes in the incidence of trachoma.
4. Antibody levels, rather than seropositivity, can be used to measure changes in disease transmission.

1.4 Study sites

This thesis includes data from seven countries: The Gambia, Lao People's Democratic Republic (Laos), Uganda, Nepal, Tanzania, Solomon Islands and Kiribati (Figure 1). Samples from The Gambia comprise the primary dataset. The datasets include countries multiple rounds of MDA have taken place: The Gambia, Laos, Uganda, Nepal and Tanzania. Of these, Nepal and Laos have been certified by the WHO as having eliminated trachoma as a public health problem, while The Gambia is preparing their dossier for elimination. The region from which the Tanzanian data were generated was shown to have no ocular Ct infection in 2002 [35]. In comparison, Kiribati was shown to have high levels of active trachoma and ocular Ct infection [36] and the Solomon Islands has moderate levels of active trachoma in children aged 1-9 years old, high prevalence of genital Ct infection [37,38] but low levels of ocular Ct infection [19]. Further information is detailed below.

Status of elimination of trachoma as a public health problem, 2018

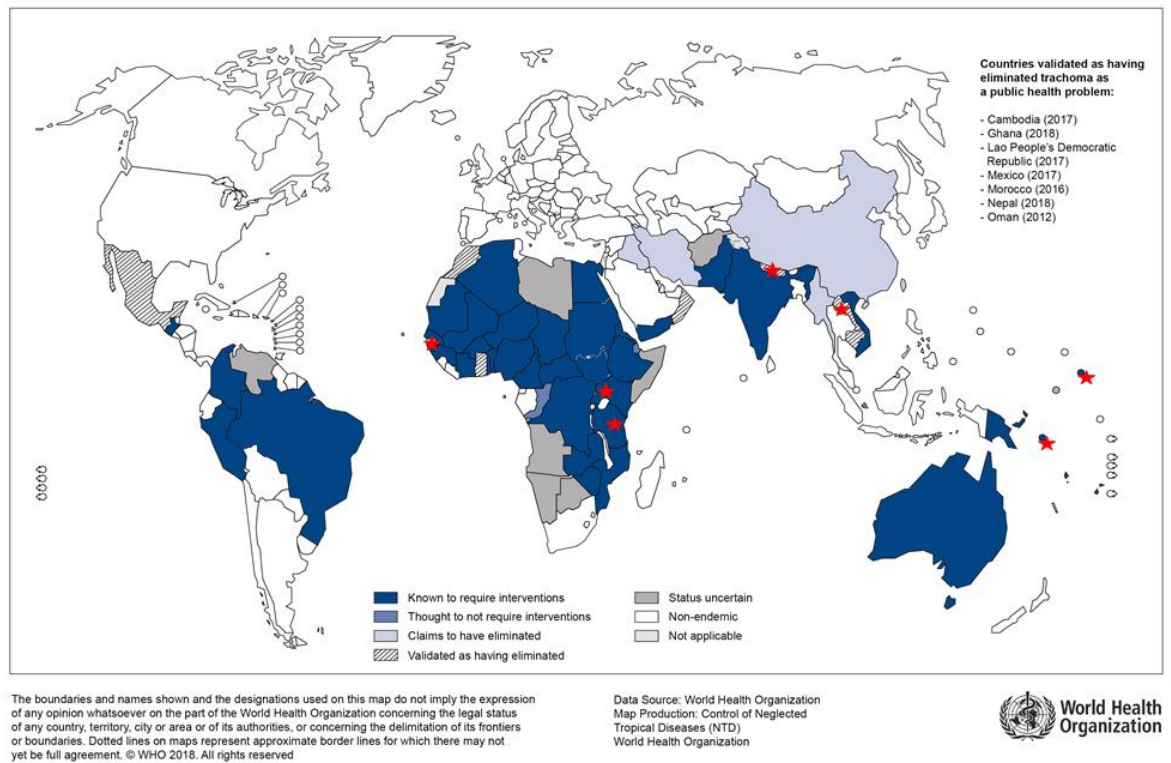


Figure 1.1. Map produced by the WHO showing the status of elimination of trachoma as a public health problem, 2018. Research sites marked as red stars. From left to right, these are: The Gambia, Uganda, Tanzania, Nepal, Laos, Solomon Islands, Kiribati. Modified from [39].

Samples from Laos and Uganda were collected by collaborators at the International Trachoma Initiative (ITI) as part of the Trachoma Alternative Indicators Study [21]. At the time of sample collection, these countries were considered to be peri-elimination, that is the prevalence of TF in children aged 1-9 years would be less than 5%, but elimination as a public health problem has not yet been validated. Little has been published about the prevalence of trachoma in Uganda [40,41], however two regions were considered highly endemic for trachoma and received three years (2010-2012) of the A, F and E components of the SAFE strategy, although no data is publicly available. DBS were collected as part of a trachoma impact assessment survey [42].

A trachoma rapid assessment (TRA) was conducted in five provinces in Laos (Oudomxay, Luang Prabang, Vientiane, Salavan, and Sekong) in 2000, finding overall that 14.8% of children examined had active trachoma [43]. A population-based prevalence study was undertaken from November 2013-August 2014 to examine 21,566 children aged 1-9 years, and 15,052 adults aged ≥15 years [43]. Three 'hotspots' were identified, where the prevalence of TF in children aged 1-9 years was above 10%. These three regions, one each in Attapu, Houaphan and Phôngsali, along with

surrounding villages were further surveyed and DBS collected during the extra survey round comprise part of this thesis [42].

The DBS from The Gambia, Uganda and Laos were assayed for antibodies against Pgp3 by the author of this thesis, who also conducted all serological analyses of these samples. These samples are described in detail in Chapter 4.

Additional serological datasets were requested and received from collaborators to compare the data from peri-elimination countries to (i) other countries or regions where trachoma has been validated as having been eliminated or where there is strong evidence to suggest that infection, transmission and disease have been eliminated, or (ii) where trachoma remains an ongoing public health problem.

Two regions in which trachoma has been 'eliminated' were also included: Nepal was validated as having eliminated trachoma in 2018 [44], while studies have shown that ocular Ct infection in Rombo district, Tanzania, was eliminated in 2005 [35]. These samples were assayed using a multiplex bead assay (MBA) and primary analyses completed by collaborators at the Centers for Disease Control and Prevention, USA (CDC) [45,46]. Secondary analyses of antibody levels were completed by the author of this thesis.

Two regions where chlamydial infections remain an ongoing health problem were included for comparison: populations in the Solomon Islands have high prevalence of genital Ct and antibodies against Ct but low prevalence of conjunctival scarring (TT) [47,48] while a recent study showed a high prevalence of disease, ocular Ct infection and antibodies against Ct in Kiribati [36]. The author of this thesis assayed the samples from Kiribati and conducted all serological analyses as well as secondary analyses of the data from the Solomon Islands.

Additional data from the Solomon Islands were provided by Dr Robert Butcher of LSHTM [48], data from Nepal were provided by Dr Diana Martin of the CDC [45], data from Kiribati were provided Dr Anthony Solomon from the WHO [36], data from Tanzania by Drs Martin and Solomon [46].

1.4.1 The Gambia

The Gambia is the smallest mainland country in Africa. It is situated in West Africa, surrounded by Senegal except for its coastline on the Atlantic Ocean. The country stretches over 400 km inland, with the River Gambia dividing the country into two nearly equal halves. The 2013 National Census estimated the population to be 1.9 million, with women comprising 51% of the total population [49]. Due to a high fertility rate (5.4 births per woman), the population structure is skewed toward young people: 42% of the population is under 15 years and about 22% are between 15 and 24 years of age.

The Gambia has a typical Sahelian climate, with a short rainy season from June to October, and a longer dry season the rest of the year.

The country became a sovereign republic in 1965, following more than two centuries of colonial British rule [50]. Administratively, The Gambia is divided into eight administrative areas: Banjul (the seat of the government), Kanifing, Brikama, Mansa Konko (formerly Lower River Region), Kerewan (formerly North Bank), Kuntaur, Janjanbureh (both formerly Central River Division) and Basse (formerly Upper River Region). To align with previously published works, in this thesis the author has chosen to refer to Mansa Konko and Basse by their historical names of Lower River Region (LRR) and Upper River Region (URR), respectively (Figure 2). For census purposes, The Gambia is divided into Enumeration Areas (EA), which cover a settlement, a cluster of small settlements or part of a large settlement; one EA generally covers 600-800 residents [51]; this is equivalent to a 'district' as described by the Global Trachoma Mapping Project (GTMP) [7].

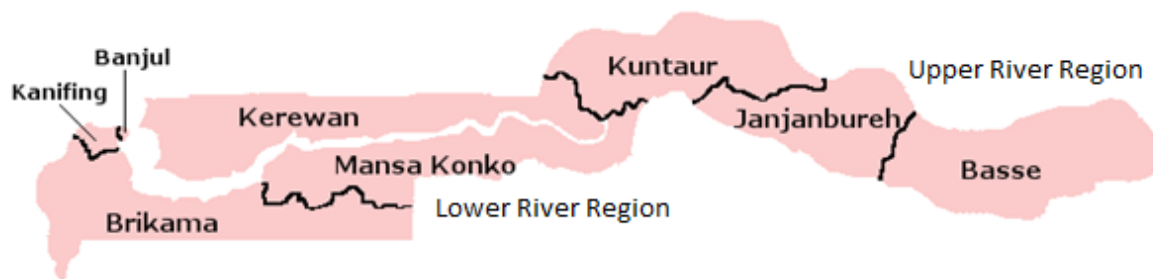


Figure 1.2. Map of The Gambia, showing current administrative areas, with the historical names provided for the two primary study districts included. Map courtesy of Wikipedia user Acntx [52].

Trachoma is associated with poverty and low access to water and sanitation services [53]. In The Gambia access to water has increased greatly, with nearly 11% of rural populations having a source of drinking water on premises and 67.2% less than 30 minutes round-trip to access drinking water [50]. Despite these improvements in water and sanitation, nearly two-thirds of rural population does not have access to improved toilet facilities, as defined by the WHO and United Nations Children's Fund (UNICEF.) Less than 13% of rural households have electricity and only 26% of rural households used soap and water for handwashing [54].

1.4.2 Trachoma in The Gambia

For many years trachoma was endemic in The Gambia. In 1986 a population-based national survey of blindness, low vision and eye disease was undertaken and found there to be a prevalence rate of blindness of 0.7%, of which trachomatous corneal opacity was responsible for 17% [55]. With only

one eye clinic and one ophthalmologist at the time, the Gambian National Eye Care Programme (NECP) was integrated into the national health services as primary health care, with support from Sight Savers International. Ophthalmic nurses are integrated into community health services and cover a population of approximately 50,000. They are overseen by senior ophthalmic medical assistants, trained in cataract surgery [56]. A second national survey in 1996 showed that the prevalence rate of blindness had decreased to 0.4% while the prevalence of active trachoma – based on the WHO classifications of TF or TI – in children aged 0-14 years dropped from 10.73% to 4.81% [57,58]. Additional NECP activities have trained health care workers in primary eye care, recognition and treatment of conjunctivitis, and surgery for trichiasis, as well as community outreach activities like school screening and face-washing [58]. Further, the NECP has developed a network of *nyateros* (Friends of the Eye), who are non-health professionals, selected by their own communities, who have been trained in good eye health practices to promote within their communities [26].

Subsequent interventions run by the National Eye Health Programme (NEHP) included the mass drug administration (MDA) of azithromycin in over 20 districts across the country between 2007 and 2010. The Partnership for the Rapid Elimination of Trachoma (PRET) [59] was embedded within the NEHP and measured the prevalence of disease and ocular Ct infection in children in four districts where MDA had been administered, including LRR, but not URR.

1.4.3 PhD Fieldwork and Samples

Primary fieldwork for this PhD research was conducted in Upper and Lower River Regions (URR and LRR, respectively) of The Gambia. URR is situated at the far east of the country, while LRR is located more centrally. I collected data and DBS over a three-month period from February to April 2014. The author assayed these DBS using a Pgp3-specific ELISA developed in collaboration with the CDC [42] and analysed all data.

Samples were also collected from Laos [43] and Uganda as part of the Trachoma Alternative Indicators Study [21]. These populations are further detailed in Chapter 4 [42]. The author assayed these samples using the Pgp3-specific ELISA and analysed all data.

Additional samples from Kiribati were provided for ELISA testing and analysis. These samples were collected as part of The Global Trachoma Mapping Project [7] undertaken on Kiritimati Island, in the far east of Kiribati, in the South Pacific [36]. Samples were collected from children 1-9 years old. The author of this thesis assayed these DBS with the Pgp3-specific ELISA and analysed the serological data.

Additional data came from the Solomon Islands, where DBS were collected from participants of all ages in participating villages in the provinces of Temotu and Rennell & Bellona as part of the Solomon Islands National Trachoma Elimination Program [48]. These samples were assayed using the same ELISA protocol as the samples above. The author performed secondary analysis of the serological data.

Data were also supplied for pre- and post- MDA populations in Nepal [45] and a post-MDA population in Rombo, Tanzania [46]. In both populations, samples were collected from participants of all ages. These samples were run on a Luminex assay at the CDC. The author performed secondary analysis of the serological data.

1.5 Study components

This PhD consists of five main components:

1. Literature review of uses of serology for monitoring Ct infection at the population-level (Chapter 2)
2. Development of a Pgp3-specific ELISA (Chapter 3)
3. Assessment of methods for determining thresholds between seropositive and seronegative populations (Chapter 4)
4. Estimation of changes in transmission rates in The Gambia using reverse catalytic modelling (Chapter 5)
5. Use of novel statistical methods to estimate age-dependent antibody levels as an alternative to seroprevalence (Chapter 6)

1.5.1 Literature Review

Historically, serology was commonly used as a diagnostic tool for genital chlamydial infections, however serology has been displaced as a diagnostic tool by more sensitive and specific methods based on nucleic acid amplification tests (NAATs), like polymerase chain reaction (PCR).

Meanwhile improvements in protein expression and purification have made serological assays more sensitive and specific for detecting antibodies which have developed as a result of previous chlamydial infection. The author conducted a systematic literature search in June 2016, which was updated in April 2018. The author outlined the antigens used, antibodies detected, methods of detection and summarised the current literature based on the public health applications of Ct serology; this includes studies of ocular and genital Ct infections.

1.5.2 Development of a Pgp3 ELISA

The author developed a Pgp3-specific ELISA with colleagues at the CDC. This was based on the existing MBA which has been previously published [33]. The author showed the ELISA to be sensitive and specific with reproducible results.

1.5.3 Assessment of threshold methods

The earlier development of a Pgp3-specific MBA relied on reference standards to set a threshold for positivity using a Receiver Operator Characteristic (ROC) curve. The 'known negative' samples were de-linked serum samples from a population of 122 children from the United States, while the 'known positives' were DBS from 11 children, residing in a region in Tanzania which had undergone three rounds of MDA, from whom ocular swabs were PCR-positive for ocular Ct infection. Of these samples, three 'known negatives' were positive for antibodies against Pgp3, while one 'known positive' was negative for antibodies against Pgp3. This highlights issues in selecting appropriate reference standards. Therefore, the author explored other internally-calibrated methods (using data generated during the study) such as finite mixture modelling (FMM) and expectation-maximisation algorithms (EM) to establish positivity thresholds between seropositive and seronegative samples and compared these to thresholds set using an ROC curve, separately maximising sensitivity and specificity and Youden's J-index, which balances sensitivity and specificity [60]. These modelling techniques are detailed in Chapter 4.

1.5.4 Estimation of changes in transmission rates

Pgp3-specific serological data were used to produce a reverse catalytic model (RCM). RCM has been previously used in studies of malaria and neglected tropical diseases [61] to determine the impact of interventions on the force of infection (Fol). The author used RCM to estimate changes in seroconversion rate (SCR) and seroreversion rate (SRR) associated with a reduction in the prevalence of trachoma in The Gambia. This method may be of use in districts where the SAFE strategy has been implemented to show a change in the Fol, and to evaluate the effectiveness of the intervention activities; one would expect to see a decrease in the Fol following the intervention.

1.5.5 Estimation of age-dependent antibody levels

Serological surveys have relied on binary seropositive/seronegative data for estimates of prevalence. Rather than dichotomise the serological data to simple seropositive and seronegative, the author used a recently developed statistical package 'tmleAb' to estimate age-dependent antibody curves in seven countries. The author of this thesis compared pre- and post-MDA data across all ages, data from children in populations where trachoma has been eliminated to those with ongoing disease

and infection and data from all ages in a population with high levels of genital Ct infections to one with low levels of both ocular and genital Ct infections.

1.6 Fieldwork Protocol

In the Gambia a cross-sectional seroprevalence survey was conducted by the author, in which 20 EAs (10 each in LRR and URR) were selected for study using probability proportional to size. Households within these EAs were then randomly selected for participation. Demographic and clinical data, photographs of everted eyelids, and finger prick blood samples- in the form of DBS- were collected from all household members who consented to participate. ELISAs were used to test for anti-Pgp3 antibodies. Seroprevalence curves were generated and compared to historical and current prevalence data.

1.6.1 Sample Size Calculations

Sample sizes were estimated based on a two-sided comparison of seroprevalence rates between the regions (surveillance Zones). If there had been significant transmission of ocular chlamydial infection in the last generation, it was anticipated that 80% of 40-year olds will have antibody, compared to a suggested 20% in settings where minimal no transmission had occurred. These are represented below as regions A and B.

The rate for each region was first calculated from the following equation:

$$P(a) = 1 - \exp(-\lambda a),$$

where $P(a)$ is the age-specific seroprevalence (prevalence at age a), and λ is the force of infection to be estimated.

Table 1.1. Estimates of seroprevalence and seroconversion rate (Λ) in two regions of The Gambia

Region	Age	Seroprevalence*	Λ
A	40	0.8	0.04
B	40	0.2	0.0056

*Estimates of seroprevalence (D. Mabey, personal communication)

Using the 'stpower' with the 'exponential' function and 90% power for a two-tailed test ($\alpha=0.05$) in Stata, the necessary sample size for comparison is 150 individuals from Region A and 810 individuals from Region B. Accordingly, we collected samples from 20% more subjects than this, approximately 1000 per region. With an average household size of 7.5 people in LRR (National Eye Health

Programme data) and 25 in URR (Grant Mackenzie, personal communication), we anticipated that 14 households should be sampled from each of the selected EAs in LRR and 4 from each of the EAs selected in URR.

These calculations assumed that the rate of seroconversion is relatively constant in the populations, that individuals, once seropositive, do not revert to seronegative and that there is no specific migration due to trachoma infection status [61].

1.6.2 Community Sensitisation

Members of the trachoma field team at the MRC Unit- The Gambia sensitised villages and obtained community-consent from the village chiefs, known as *alkalos*. Household head lists were compiled with the assistance of each *alkalo* and a census conducted in sequential randomly selected households consenting to participate until their combined population exceeded the projected sample of 100 per EA by 50%. Team members and eye health nurses explained the study to each head of household, answered any questions and explained the written consent form. All residents of selected households were invited to participate. If a selected household chose not to participate, the next one on the list was approached.

1.6.3 Inclusion and Exclusion Criteria

Inclusion Criteria

Resident in the selected village, a member of a selected household, willing to consent and participate; if younger than 17, a guardian must have been willing to provide consent. All residents of selected households were invited to participate. Assent was sought from participants aged 12-17.

Exclusion criteria

We invited all residents of selected households to participate. Age was not an exclusion factor. Participants were able to stop participating at any point during the process. Some selected households were 'reserves' and were not approached to participate if the projected sample size had been reached.

1.6.4 Informed Consent

Written (thumbprint or signature), informed consent was obtained from each adult participant and each participating child's parent or guardian. Each participant was free to ask questions to the field team before providing consent and throughout the ocular examination and sample collection.

Assent was sought from children ages 12-17.

1.6.5 Data and Sample Collection

The survey in the Gambia was undertaken between February and April 2015. Individual-level data were collected by a local eye-health nurse, including gender, age (in years or as date of birth) and ethnic group. Facial cleanliness was observed by a trained trachoma grader before each eyelid was everted for grading and photography. Demographic information, facial cleanliness and trachoma grade were recorded on a standardised form by the author (Appendix 2). Forms were entered into a custom built EyeScores database [62] to link with photographs of everted eyelids. A fingerprick blood sample was collected onto filter paper by a second eye health nurse and allowed to dry for approximately 5 hours; each filter paper had six extensions, calibrated to absorb 10 μ L of blood. All samples were placed in individual plastic bags, then stored with desiccant in larger sealed plastic bags. Samples were transferred to the MRC Unit-The Gambia where they were stored at -20°C . All samples were subsequently shipped to LSHTM for ELISA testing.

1.7 Laboratory testing

DBS were tested for IgG antibodies against Ct using a Pgp3-specific ELISA. A detailed protocol is provided in Chapter 3. In brief, DBS were eluted into 250 μ L of phosphate-buffered saline (PBS) with 2.5% w/v milk powder plus 0.3% v/v tween 20 (PBSTw-milk). Immunlon 2HB plates were coated overnight at 4°C with 50 ng/well glutathione S-transferase (GST)-tagged Pgp3 then blocked for 1 hour with PBST the following day. Plates were incubated for 2 hours with 50 μ L of elution mixture and incubated at room temperature with HRP-labelled mouse anti-human IgG for 1 hour. Plates were then incubated for ten minutes in the dark with tetra-methylbenzidine (TMB), stopped with 50 μ L 1N H_2SO_4 and optical density was read at 450 nm (OD_{450}). Control sera with known ratios of Pgp3 antibodies (1000 units, 500 units, 200 units, 50 units and negative control serum) and a blank consisting of PBSTw-milk were run on every plate. All specimens were normalised to the 200 unit control sera, which typically gave a reading of 1.0 OD_{450} .

1.8 Data Analysis Tools

All statistical analyses were carried out using R software version 3.3.2 [63]. Details of specific analyses for each component of this thesis are highlighted in the individual data chapters following.

In brief, Chapter 4 considers 4 methods of setting seropositivity thresholds. These include three internally calibrated methods, relying solely on data generated in the study to establish thresholds; these are then compared to the commonly ROC curve which has been frequently used in trachoma serological studies [18,33,46,64–67]. Using a ROC curve to set a threshold requires well-

characterised positive and negative reference samples from the population of interest. Inter-laboratory calibration is also required, ideally using the original reference specimens. Misclassification of the reference samples can result in an inappropriate threshold.

The FoI for two populations in The Gambia was modelled in Chapter 5. This work involved estimating seroconversion and seroreversion rates based on catalytic models [68] and has previously been applied to both malaria [69–71] and Chagas disease [61].

Finally, an analysis using antibody acquisition models is presented in Chapter 6, in which changes in community antibody profiles are compared between pre-, peri- and post-elimination countries and between communities where genital chlamydia is highly prevalent to one where both ocular and genital chlamydia infection levels are low.

1.9 Ethical Considerations

This thesis was conducted in accordance with the Declaration of Helsinki. Ethical approval was obtained from The Gambia government/Medical Research Council (MRC) Joint Ethics Committee (SCC1408v2) and the London School of Hygiene and Tropical Medicine (references 6319, 6514, 8355, 8918). For specimens not directly collected by the author, ethical approval was obtained from

- Laos: the Ministry of Health of the Lao People’s Democratic Republic (No:48 NIOPH/NECHR),
- Uganda: Ugandan Ministry of Health (VCD-IRC/053)
- Tanzania: Centers for Disease Control and Prevention (USA), Kilimanjaro Christian Medical College (KCMC)/ Tumaini University, and the National Institute for Medical Research (TZ) [46]
- Nepal: Children’s Hospital and Research Center Oakland (IRB number 2013-043) and by Nepal Netra Jhoti Sangh (Nepali Prevention of Blindness Program) [45].
- Kiribati: London School of Hygiene & Tropical Medicine (reference numbers 6319, 8355 and 10136) and the Kiribati Ministry of Health and Medical Services (08/11/2015) [36]
- Solomon Islands: London School of Hygiene & Tropical Medicine (LSHTM; 8402) and Solomon Islands National Health Research Ethics Committee (HRC15/03)

1.10 Data Management

Each participant was assigned a unique identifying number, which was used to link photographs and demographic, clinical and laboratory data. In The Gambia, all fieldwork information was collected on standardised forms, which were then double entered into a custom build database, EyeScores [62]. Data from the other sites were provided directly to the author from collaborators.

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Chapter 2- *Serological tests for the surveillance of Chlamydia trachomatis infections: the history, development and review of their use in public health*



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SECTION A – Student Details

Student	Stephanie Michelsen
Principal Supervisor	David Mabeu
Thesis Title	Chlamydia trachomatis serology as a means of monitoring intervention activities to eliminate trachoma as a public health problem

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	PLOS ONE		
When was the work published?	2017		
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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I collected the samples from the Gambia, performed the ELISA on all the samples, conducted the analyses, wrote and edited the paper and responded to reviewers
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Student Signature:  _____
Supervisor Signature:  _____

Date: 10/09/18
Date: 12/9/18

Summary

The bacterium *Chlamydia trachomatis* (Ct) causes both genital and ocular infection. Clinical signs are used to diagnose trachoma, caused by ocular infection with Ct, while genital infection is diagnosed using nucleic acid amplification tests (NAATs) to detect the presence of chlamydial DNA. Serology has historically been used as a diagnostic tool for both ocular and genital infections, however this is not an appropriate diagnostic method. We summarised the historical context of chlamydia serology reviewing samples, testing methodology, antigens, immunoglobulins detected and methods of analyses. We reviewed published serological studies to provide an overview of the current landscape of Ct serology as it relates to public health.

Methodology

The author of this thesis performed a systematic literature search of online databases (PubMed, Cochrane, Lilacs, Scielo, Scopus and Web of Science) in June 2016, and updated it in April 2018, following PRISMA guidelines. Studies were limited to peer-reviewed Ct serology studies in the public health context, that is not for research or individual diagnostics purposes with the reported outcome being the prevalence of antibodies against Ct. The studies were heterogeneous in terms of population studies, sample collected, assay and antigen used and method of determining positivity.

Conclusion

Overall, 24 studies were included: 9 trachoma-related studies and 15 studies of genital Ct infections. The diversity of studies prompted a review of the historical development of serological assays for Ct to provide a more thorough overview of the elements of Ct serology and its public health applications. Few head-to-head comparisons have been made comparing either commercial and in-house assays to each other or comparing different antigens, assay types or threshold methods across platforms. A standardised set of reference standards should be produced to facilitate comparisons between assays and settings if serology is to become more widely accepted as a useful public health tool.

In the subsequent chapters, the author will cover the development of a Pgp3-specific ELISA for detecting antibodies against Ct to be used to measure seroprevalence and antibody acquisition in populations where trachoma is or has been a public health problem. Data generated using this ELISA are used to estimate changes in the force of infection following public health interventions.

Serological tests for the surveillance of *Chlamydia trachomatis* infections: the history, development and review of their use in public health

Authors: Stephanie J Migchelsen, Sarah C Woodhall, Becca Handley, David Mabey, Chrissy h Roberts

2.1 Introduction

Chlamydia trachomatis (Ct) is one of the most frequent causes of bacterial sexually transmitted infections (STI) [1], as well as being the leading infectious cause of blindness [2]. The species consists of a number of serovars. To some extent it is possible to link serovars to pathology and tropism, with types A, B, Ba and C having tropism to the eye and causing trachoma. The remaining serovars are generally found in the genital tract, with types D-K causing the classical genital chlamydial infection whilst types L1, L2 and L3 cause a more severe form of disease known as lymphogranuloma venereum (LGV) [3].

Genital infection with Ct is common worldwide but ocular infection is now predominantly limited to tropical developing countries [4]. Repeated or prolonged infections (both genital and ocular) lead to complications and pathological sequelae. Genital Ct infection can cause pelvic inflammatory disease (PID), with potential sequelae of tubal factor infertility (TFI), ectopic pregnancy, chronic pelvic pain and infertility in women [5–8]. In men, genital Ct infection can cause epididymitis, an inflammation of the epididymis which causes severe testicular pain [9,10]. There is also some evidence that current infection with Ct is linked to sperm DNA fragmentation and reduced fertility [11]. Babies born to infected women are at risk of ophthalmia neonatorum or bacterial pneumonia [12].

Following ocular infection with Ct, raised follicles develop on the upper tarsal conjunctiva, known as trachomatous inflammation- follicular (TF). This is often followed by inflammatory thickening, called trachomatous inflammation- intense (TI). The long-term sequelae of this inflammation include scarring of the upper tarsal conjunctiva, leading to trichiasis, a condition where the eyelashes rub against the surface of the eye, which can lead to corneal opacity and blindness [13].

Methods for understanding the burden of Ct in populations differ between ocular and genital chlamydia disease. The prevalence of trachoma is determined by the presence of clinical signs detected by everting the upper eyelid to examine the tarsal surface [14]. Cross-sectional prevalence studies are typically performed in endemic locations, with children aged 1-9 years in selected villages being examined. Studies have shown a disparity between the clinical signs and the presence of Ct infection, particularly in areas of low-endemicity or post-intervention communities, with clinical signs often persisting after infection has been treated [15,16]. The prevalence and incidence of Ct infection is challenging to measure as infections are often asymptomatic and self-clearing [17]. In the primary care context, nucleic acid amplification tests (NAATs) are used to test a variety of specimen

types, including urogenital, pharyngeal and rectal swabs, as well as urine specimens [18] and these tests are also amenable to use in population monitoring and prevalence surveys. Some previous studies have been based on patients attending sexual health clinics, which suffer from population selection bias, selecting those at higher risk of chlamydia [19,20] whereas studies based on representative sampling show lower prevalence [21–23].

Unlike a clinical diagnosis based on signs and symptoms, or a molecular diagnosis based on genetic material, serological assays detect antibodies against specific antigens and are not commonly considered a diagnostic tool for current infection, either ocular or genital, as antibodies persist for a time once the infection has self-cleared or has been treated. Tests for infection and disease prevalence do not account for past exposure, nor do they measure the cumulative exposure to infection that lead to the sequelae that intervention and screening programmes for trachoma and genital Ct infection aim to minimise. Serology serves an altogether different purpose and is not a replacement for clinical or molecular diagnostic tools: NAATs are the recommended diagnostic tool for current infection [24,25]. The presence of antibodies can be used to indicate past exposure and changes in transmission [26,27] as well as indicating the development of severe pathology and scarring sequelae [28].

Historical reviews have focused on the use of Ct serology as a diagnostic test [29,30] but few recent works provide a detailed description of how these methods can be turned towards applications in programmatic surveillance for disease control [31]. A recent upsurge in interest in such applications has led us to review the use of anti-Chlamydial serological tests as tools for surveillance, drawing attention to particular studies in which serology has been used to measure the prevalence of anti-Ct antibodies in population-based samples. We present a historical context and examine some of the technical aspects of using serology. We show the challenges and opportunities of using serology to detect antibodies against Ct in the context of programmatic surveillance and disease control.

2.2 Technological platforms

Serological testing for *Chlamydia spp.* has a history dating back almost a century, with early (c. 1935) technologies such as the Complement Fixation (CF) assay having been largely replaced by the microimmunofluorescence (MIF) test and Enzyme Linked Immunosorbent Assays (ELISA) in the 1970s. Whilst ELISA remains a mainstay of serology in the 21st century, the technology has continued to evolve as the hardware has become more automated and the quality of the purified antibodies has increased over the years. In the last ten years it has become possible to adapt the principles of ELISA to highly multiplexed assays, using solid state micro-bead technology to simplify workflows and increase throughputs, whilst also improving precision and accuracy of measurement. Full proteomic analysis via microarray-based studies has also become both possible and affordable and has led to a paradigm shift in quantitative multi-antigen serological analysis. In this section we briefly introduce key studies that have used these technologies in Ct research.

2.2.1 Complement fixation (CF)

The first serological assay was published by Bedson in 1935 and was a complement fixation (CF) assay using a group-specific lipopolysaccharide (LPS) antigen [32]. CF was first used to diagnose cases of human psittacosis, caused by *C. psittaci* and LGV [32]. However, because it detected genus-specific *Chlamydia* lipopolysaccharides, it had poor specificity due to cross-reactivity [33,34]. Conway et al. used CF to detect high titres of antibodies in women with a history of PID and tubal damage[35]. Yet, it was not specified if the PID was due only to Ct, as other genital infections, such as gonorrhoea and bacterial vaginosis can cause PID[36,37]. Serological evidence of Ct infection was also detected in pregnant or potentially fertile women. Thus, although a positive CF result was correlated with tubal damage, it was not diagnostic of the condition, as many women without tubal damage had serological evidence of chlamydial infection [35]. Similarly, Richmond and colleagues performed a study in which CF was used to demonstrate anti-Ct antibodies in patients with psittacosis and those attending venereal disease and family planning clinics [38]. (Figure 2.1)

Complement fixation

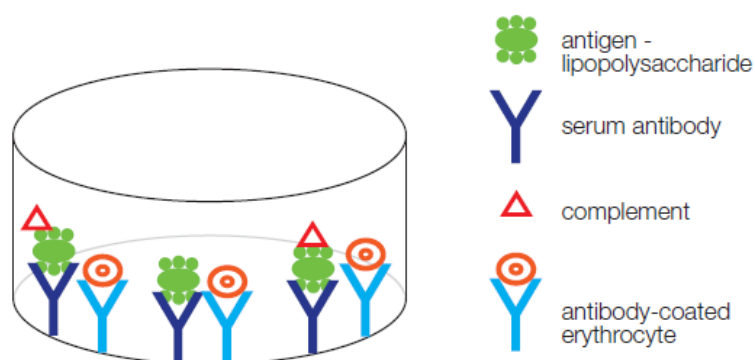


Figure 2.1 Schematic representation of Complement Fixation. If the patient's serum contains antibodies against the antigen of interest (lipopolysaccharide), they will bind to the antigen to form antigen-antibody complexes. The complement proteins will react with these complexes and be depleted. Thus, when the antibody-coated erythrocytes are added, there will be no complement left in the serum. However, if no antibodies against the antigen of interest are present, the complement will not be depleted and it will react with the antibody-coated erythrocytes, lysing the erythrocytes and spilling their contents into the solution, thereby turning the solution pink.

2.2.2 Microimmunofluorescence (MIF)

In 1970, Wang and Grayston developed the microimmunofluorescence (MIF) test to serotype strains of *C. trachomatis* [39], having already raised a range of type-specific polyclonal antibodies, which allowed for serotyping of many of the serovars [40] using elementary bodies (EB). EBs are non-replicating infectious chlamydial particles that are released when an infected host cell ruptures [41]. Serovar-specific EBs are fixed onto glass slides as distinct dots and an immunoreaction is detected if a clinical specimen contains antibodies against the antigen. The reaction is visualised with a secondary antibody labelled with fluorescein isothiocyanate (Figure 2.2). The use of a specific secondary antibody allows the identification of antibody isotypes (commonly IgG, IgA or IgM). Both CF and MIF allow for serial dilutions to determine end-point titre of a specimen. Wang and colleagues also demonstrated serovar-specific IgM and IgG in serum and tears [42], as well as correlating the presence of IgM with infection [43]. It should be noted that the MIF is a labour-intensive in-house method, with modifications common [44,45], therefore results are variable in terms of both quality and performance [30]. For many years, MIF was considered the only specific and sensitive serologic assay for any of the *Chlamydia* spp. [29,46], however, both its sensitivity and specificity have been questioned [47–49]. Peeling and collaborators demonstrated the inter-

laboratory variability of the assay, which makes the comparison of published data from different labs challenging and underlines a need for standardised serological tests [50].

Micro immunofluorescence

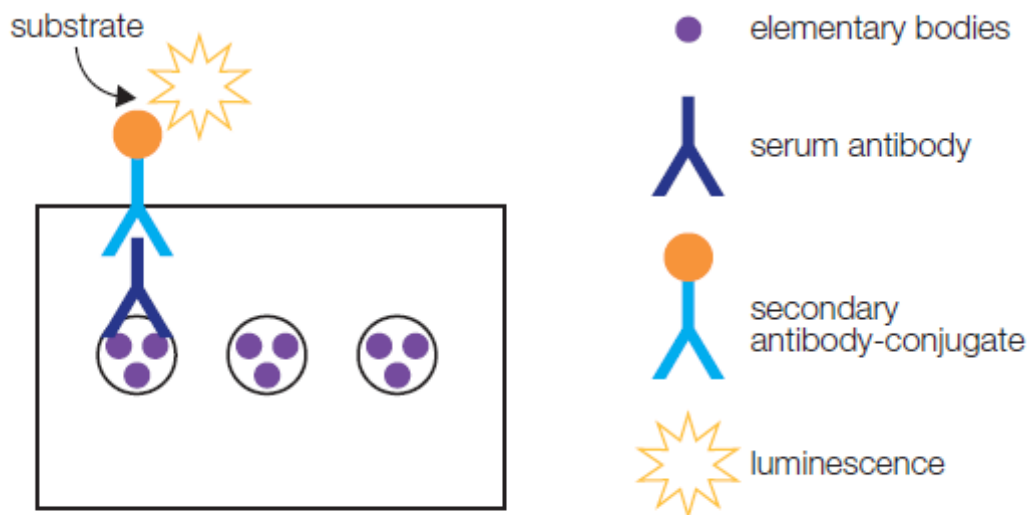


Figure 2.2. Schematic representation of the microimmunofluorescence (MIF) assay. Chlamydial elementary bodies (EB) are fixed onto a glass slide, a clinical specimen is applied. If anti-EB antibodies are present, an immunoreaction can be detected by applying a secondary antibody labelled with fluorescein isothiocyanate.

2.2.3 ELISA

Enzyme-linked immunosorbent assays (ELISA) were developed with advances in diagnostic technology, such as antibody purification techniques. They were easier to perform than MIF, more objective [51] and large batches of samples could be processed simultaneously using 96-well plates. In brief, antigen is coated to the bottom of the wells, sample is applied, whereupon specific antibodies will bind to the antigen. A conjugated secondary antibody is applied and detected using a fluorescing substrate (Figure 2.3).

Numerous ELISAs for the diagnosis of chlamydial infections were introduced in the 1980s [52–55]. These ELISAs commonly used elementary bodies (EBs) as antigen [56]. These ELISAs were genus-specific, rather than species-specific and were unable to distinguish between antibodies for Ct and *Chlamydia pneumoniae* (Cp), a common human respiratory pathogen [16]. An appropriate screening test for chlamydial seroprevalence must be able to distinguish between species. With the advent of

protein recombination techniques, new ELISAs have been developed providing greater reported sensitivity and specificity against MIF and CF [57,58]. Although most are indirect ELISAs, a double-antigen “sandwich” ELISA has also been developed and shows increased sensitivity when compared to some commercial indirect ELISAs [59,60].

An assay that detects antibodies to non-species-specific antigens could lead to over-estimation of the prevalence of Ct infections. In trachoma-endemic populations, this may lead to prolonged elimination activities and excessive use of antibiotics.

Indirect ELISA

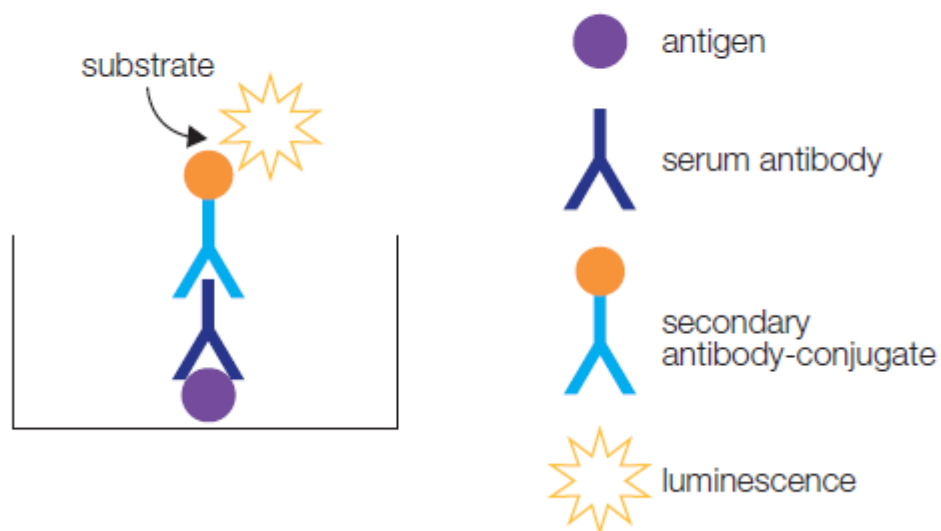


Figure 2.3. Schematic representation of the indirect enzyme linked-immunosorbent assay (ELISA). Immunogenic chlamydial proteins (antigens) are coated to the bottom of wells, a clinical specimen is applied and antibodies specific to the antigen will bind. The immunoreaction can be detected by a secondary antibody conjugated with a fluorescein conjugate.

2.2.4 Multiplex bead assay (MBA)

An antigen-based multiplex bead assay (MBA) has been recently developed and allows for the identification of antibodies against multiple antigens [61]. Fluorescent microspheres are conjugated to antigens (Figure 2.4), and the varying spectral addresses of the labelled beads allow for up to 100 bead-antigen combinations to be used in a single assay well [62]. Following a workshop by the World Health Organization (WHO), experts in neglected tropical diseases (NTDs) concluded that antibody-based MBAs offered the greatest prospect of simultaneous monitoring of elimination efforts for

several NTDs, for instance trachoma, lymphatic filariasis, schistosomiasis, onchocerciasis, and soil-transmitted helminth infections [63]. The multiplex nature of the assay allows for dozens of antigens to be included, evidence of infection with several organisms to be obtained and for each sample to be assayed with several antigens. Previous studies using the MBA have used beads conjugated with plasmid gene product 3 (Pgp3) and CT694 (see Antigen selection, below), two immunogenic proteins produced by Ct, to monitor the changes in seroprevalence in trachoma-endemic populations at baseline and six months or one year after a single round of mass drug administration (MDA) [64,65], as a measure of the transmission intensity [26,66] and as a proxy for the force of infection (FoI) using seroconversion rates [27]. These studies have shown that in a programme-monitoring capacity, serological studies are a reasonable proxy for infection or clinical signs, particularly in areas of low-endemicity or post-MDA communities.

Multiplex bead assay

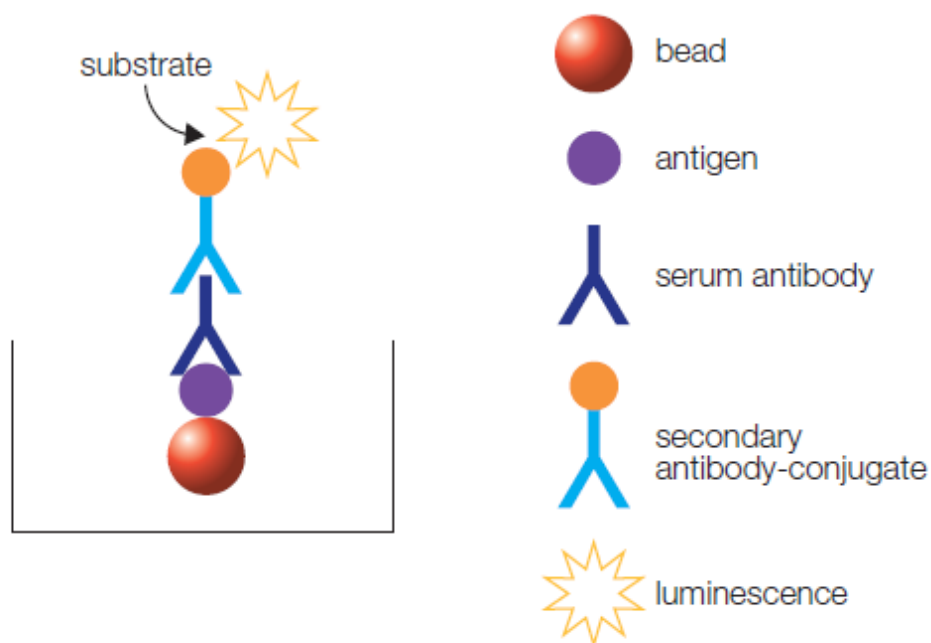


Figure 2.4. Schematic representation of the multiplex bead assay. Antigens are coated to fluorescent microspheres (beads) to which specific antibodies in clinical specimens will bind. A secondary antibody-conjugate is then applied and visualised by reading light emitted when the beads are excited using laser technology.

2.2.5 Proteomics

Advances in recombinant techniques and proteomics have led to refinements in serological techniques. This, in turn, has led to an exploration of potential immunological targets using whole genome scale proteomic arrays, with several immunogenic antigens being identified. The development of synthetic and recombinant protein methods allowed for improved ELISAs, using more Ct-specific antigens- such as the major outer membrane protein (MOMP), heat shock proteins (hsp), Pgp3 and CT694.

By assaying the peptides expressed by the open reading frames (ORF) of the chlamydial genome and plasmid, researchers have been able to identify antigens that elicit an immune response in infection-positive women as well as pathogenic antigens that are specific to different scarring pathologies. Wang et al. performed a whole genome-scale proteome array of Ct serovar D: they expressed peptides encoded by the genome and plasmid and assayed them using a microarray against sera from Ct-infection positive women, with sera from infection-negative women as controls [67]. Of these proteins, 27 were recognised by $\geq 50\%$ of the sera and were thus designated immunodominant antigens. These included antigens localised in the membrane, like MOMP, OmcB, and PmpD; proteins in the inclusion membrane, as well as proteins secreted into the host cell cytosol, such as Pgp3 [68]. The same whole genome array was used to profile antibody responses in individuals with and without trichiasis in The Gambia [69]. This study identified ten immunodominant antigens, including Pgp3, and CT694. Four additional antigens were associated with the trichiasis phenotype, producing a reaction only, or more strongly, in individuals with trichiasis compared to the controls who had no trichiasis. Another genome-wide study by Rodgers and colleagues was conducted using samples from women with laparoscopy-confirmed TFI and infertile women without TFI [70]. Many antigens elicited antibody responses in both groups, however 10 antigens were uniquely identified to elicit a response in TFI patients (100% specificity). Of these, two antigens (CT443 and CT381) had a sensitivity of 67.7% against clinically-diagnosed TFI, suggesting that they may prove to be useful biomarkers in the differential diagnosis of TFI. As such, serological assays based on these antigens may prove a less invasive tool than hysterosalpingogram or laparoscopy for diagnosing tubal pathology [71]. From the studies above, it is clear that the usefulness of serology as a tool depends on the careful selection of antigen(s) and also on the particular disease phenotype that is to be diagnosed.

2.2.6 Summary of Diagnostic Platforms

The use of a multiplex assay allows for the detection of antibodies against multiple antigens. This can be used to detect co-morbid diseases in integrated surveys, as has previously been demonstrated for

yaws and trachoma [72] and for a panel of infections endemic to Haiti, including lymphatic filariasis, giardiasis, toxoplasmosis, measles and malaria, as well as filarial antigens [62]. This type of multi-antigen panel has been recommended by experts as the ultimate goal in NTD control and elimination [63]. Multi-antigen panels could also be used for detecting biological markers of upper genital tract infection and disease sequelae if they were to be used for monitoring the sequelae of genital Ct infections and estimating the risk of infertility. It is likely that a panel of antigens will be more informative than a single antigen assay, as suggested by microarray studies [69,70,73]. MBA platforms are more expensive than an ELISA plate reader, which may put them out of reach for public health programmes in low- and middle-income countries, in which case, the alternative may be to use a single-antigen ELISA assay. Both the MBA and ELISA are more objective and less labour-intensive than MIF and can be automated or semi-automated.

2.3 Isotype selection

The isotype of an antibody is determined by the heavy chains and although five isotypes exist in humans, three are of primary interest: IgM, IgA and IgG. These isotypes emerge at different times and will persist for varying lengths of time following infection (Figure 2.5).

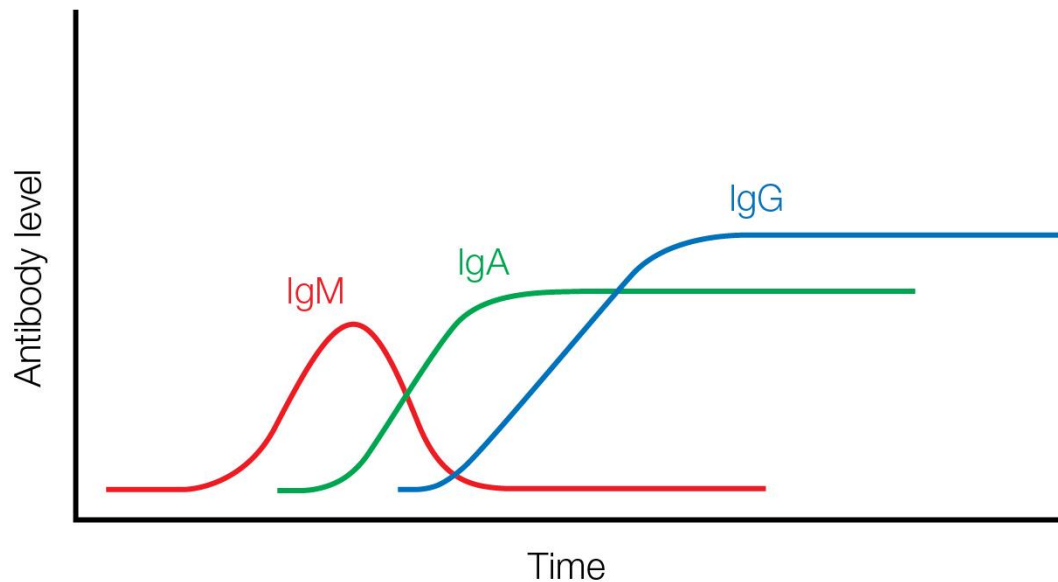


Figure 2.5 Pattern of antibody kinetics following infection with Ct. IgM is the first antibody to appear and this response is short-lived. IgA and IgG appear following the infection and persist longer.

In serological assays, serum antibodies are bound to fixed antigens, which are then detected using a tagged secondary, anti-human antibody. Any serum antibodies specific for the fixed antigen will bind to it; these are then detected using anti-human antibodies specific for one of the three isotypes of interest. Thus the selection of secondary antibody is critical in detecting the antibodies that form the immune response.

2.3.1 Immunoglobulin M (IgM)

IgM is the first antibody produced in response to an initial exposure to an antigen. Following primary chlamydial infection, IgM titres increase within two-four weeks and are lost within two-six months [46,74,75]. Although the presence of anti-Ct IgM has been found in the sera of patients with suspected or proven Ct infection [76], its production is short-lived and only a small proportion of people infected by Ct have been shown to have IgM antibodies against Ct [77]. In a study by

Trehan et al., of the 35 women who were culture-positive for genital Ct infection, only 49% had detectable levels of IgM [45]. Because chlamydial infection is often asymptomatic, patients may not present until their levels of IgM have dropped below detectable levels [77]. Similarly, in patients with trachoma, Rahi et al. demonstrated that anti-chlamydial IgM correlates with clinical inflammation but elevated levels of IgM were rare, and in patients with severe trachoma no IgM was detected [78]. For this reason, IgM is not a practical immunoglobulin to measure in serological assays.

2.3.2 Immunoglobulin A (IgA)

Immunoglobulin A (IgA) is induced by the presence of microbes and is produced by plasma cells [79]. It is the principal immunoglobulin of mucosal membranes, binding to polymeric receptors expressed by mucosal epithelial cells [80]. It is common in areas such as the urogenital tract and epithelium of the conjunctiva, both areas for which Ct exhibits tropism.

Goodhew and colleagues used the MBA to measure the IgG and IgA immune responses to Pgp3 and CT694 in serum from children living in a trachoma-endemic region in Tanzania [64]. Fewer children had IgA responses than IgG responses, a difference more pronounced in older children; the measured levels of antibody were also lower which was expected, considering the lower levels of IgA in serum. Levels of IgA in the serum are related to total antigen dose, so the lower seroprevalence of IgA may indicate a lower infectious load [64]. In a study by Ghaem-Maghani and colleagues involving 113 patients from a genitourinary medicine (GUM) clinic, the IgA response was more prominent in patients with uncomplicated genital Ct infections compared to the groups with complications. Cervical IgA levels measured were 30-50 times higher than the levels in serum at baseline and both cervical and serum levels of IgA were higher than IgG levels [81]. The authors suggested that mucosal IgA may be indicative of recent infection, consistent with the plasma cells that are attracted to the site of infection, as shown in a study by Crowley-Nowick and colleagues [82]. Theoretically, IgA levels may provide evidence of a recent or ongoing infection [83]; however, due to the low levels of IgA present in the blood, it seems unlikely that future serological assays will be targeted to detect it. If more accurate assays were developed using mucus or tears, an IgA-specific assay may be worth revisiting.

2.3.3 Immunoglobulin G (IgG)

IgG is the most common type of antibody found in sera and represent approximately 75% of serum antibodies in humans [84]. IgG is produced by plasma B cells as part of the humoral immune response. Upon infection, IgG levels rise within six weeks and usually decrease slowly [85], making

them an ideal indicator of past infection; both trachoma and genital chlamydia studies suggest that IgG persists for months and possibly years after infection [64,86,87]. Numerous studies have used the presence of anti-Ct IgG as a proxy measure for the transmission of Ct infection in population-based studies [27,59,92–101,60,102,103,64,66,72,88–91] as well as using it as a tool to estimate the proportion of disease sequelae attributable to Ct infection [104–110].

2.3.4 Summary of Isotype Selection

Both IgM and IgA are of limited value in serological assays: IgM persists for only a short time, while IgA is scarce in serum samples [111]. IgG is the most practical antibody for serological studies as it is the most common immunoglobulin in serum[84] and persists following clearance of the bacterium. Compared to the other immunoglobulins mentioned above, IgG is the most commonly studied antibody in the serological studies presented in this review.

2.4 Antigen selection

The core functionality of any serological test is provided by highly specific and highly avid interactions between a purified antibody and its corresponding antigen. Bound antigen is used as the instrument to detect circulating antibodies. These antibodies, if detected, are evidence of either a current or prior immune response to a Ct infection.

Human immune responses are highly heterogeneous at both individual- and population-level and the appropriate selection of antigens is therefore a crucial determinant of the value of the serological test. The ideal antigen choice is one or more peptides or proteins that can be shown to induce strong immune responses in the majority of persons exposed to infection and do not cross react with other infectious agents. In this section we describe and assess the use of various Ct antigens throughout the literature.

2.4.1 MOMP

The major outer membrane protein (MOMP) is a 39kDa protein encoded by ORF CT681 [112]. It has long been recognised as an immunodominant protein [113], and although there is considerable amino acid homology between proteins encoded by Ct and Cp, it appears that cross-reaction between Cp anti-MOMP antibodies and anti-Ct MOMP antibodies is rare [114,115]. A study by Närvänen et al. [116] used Ct-synthetic peptides derived from the variable domain of MOMP in an ELISA to detect antibodies against *C. trachomatis*. Samples from children with Cp antibodies determined by MIF did not show any reactivity in the ELISA [116]. However, at least one study has shown conflicting results [117]. Recombinant MOMP antigen has been used by Mygind and colleagues to detect anti-Ct antibodies with a reported sensitivity of 80% and specificity of 91% [118] when compared to the MIF. When used to detect anti-Ct IgG in sera collected from patients with acute Ct urogenital infection determined by NAAT and healthy blood donors, sensitivity was 61% and specificity 84% [119]. This suggests that MOMP is not immunoreactive in all participants or there was insufficient time between infection and testing to detect MOMP-specific IgG, which typically increases six weeks-post infection [85]. An additional study by Morré et al. compared three MOMP-specific ELISAs against MIF [120]. Sera from 149 women were analysed for anti-Ct IgG and IgA. Cervical screens were PCR-tested with 43 women positive for Ct DNA, and 106 women were PCR-negative. For all four assays, the overall seroprevalence rate was 40% for IgG and 7% for IgA. In infection-positive women, the IgG seroprevalence rates were two to three times higher than infection-negative women. Not all PCR-positive women were seropositive (74% sensitivity), nor were all PCR-negative women seronegative (75% specificity). An additional study by Miettinen and colleagues looked at the presence of MOMP-specific IgG and IgA in women with acute PID [113].

When compared to culture, IgG antibodies had 53% sensitivity and 89% specificity. The authors note that anti-MOMP IgG was frequently detected in women with PID who were not culture-positive for Ct and among apparently healthy controls, most likely reflecting previous Ct infections. Similar results were seen in studies with patients with TFI and subfertility [118,121]. These studies highlight that infection is not an appropriate reference standard for a serological assay.

2.4.2 Heat shock protein (hsp)

Heat shock proteins are a family of highly-conserved proteins common to both prokaryotes and eukaryotes; bacterial hsps are highly immunogenic targets in humans [122]. They are produced by cells in response to stressful conditions, such as changes in temperature or exposure to UV light. Three chlamydial hsps (chsp) have been studied. Chsp70 has been identified as an in-vitro target of neutralising antibodies [123–125] whilst chsp10 and chsp60 (which are co-expressed) [126] both have important roles in eliciting the host immune response [101,127]. These chsps are highly conserved in chlamydial species with greater than 95% within-genus protein-level homology [122]. They also have approximately 60% amino acid homology with hsps from other bacterial species and approximately 50% homology with human hsps [122]. Numerous studies have shown a relationship between serum IgG antibody response to hsp60 and the development of scarring sequelae following genital Ct infection [101,125,128–132]. A similar, albeit more limited, response is seen in cases of trachomatous scarring, with the presence of chsp-specific serum IgG associated with conjunctival scarring in a study of 296 children and adults in The Gambia by Peeling and colleagues [28]. In a study by Patten et al., IgA against chsp60 was significantly correlated with acute chlamydial infection, whilst anti-chsp60 IgG was significantly correlated with confirmed PID [133].

2.4.3 CT694

CT694 is a small protein of approximately 40 kDa [112]. It is membrane-localised [134] and thought to play a role in host cell invasion or subversion of the immune system [135]. During early infection of the host cell, CT694 is secreted by the type III secretion system [136]. Early studies found that cross reactivity between antibodies against Ct and Cp lead to high rates of false positivity [137–139]. Although there are no known homologous sequences in the genomes of chlamydial species [136], some epitopes of CT694 may be structurally homologous to epitopes of other Gram-negative bacteria [30]. An MBA specific for CT694 and Pgp3 against *C. trachomatis* antigens has been developed by Goodhew et al., and used in numerous trachoma-endemic and previously-endemic areas [27,62,64,66,99,100]. CT694 has not been used in any single-antigen assays to-date as it may

produce variable results over time [64]. CT694 shows similar levels of sensitivity (91%) and specificity (98%) as Pgp3 when compared to PCR used as a reference standard. In post-MDA communities, and at an individual level, CT694 antibody response correlated well with both clinic signs of infection and PCR positivity [99]. In an additional study that measured antibodies against CT694 in children aged 1 – 9 years of age at baseline and 6-months post-treatment, antibody levels decreased but none of the children reverted to seronegative status [64].

2.4.4 Pgp3

Plasmid gene product 3 (Pgp3) is encoded by the Ct plasmid, which is not present in Cp, making the antibody response specific to Ct [140]. The Pgp3 antigen is recognised by specific IgG [141] and is thought to be the most immunodominant antigen encoded by the Ct [67]. Pgp3 has been used in numerous serological assays for both ocular [72,98,99,103,142] and genital Ct infection [57,102,143,144] as a proxy measure for the impact of intervention and screening programmes. Both commercial and in-house Pgp3-specific ELISAs have been developed, with varying sensitivity and specificity against MIF, clinical disease and infection as measured by PCR, depending on the population used to characterise the assays [57,103,143]. A Pgp3-specific in-house ELISA developed by Wills et al. was evaluated against three commercial ELISAs for IgG against MOMP and one MIF assay for IgG and IgM against EBs [57]. The sensitivity and specificity were determined using sera from both female and male GUM clinic-attendees who were diagnosed with Ct infection at least one month prior to serum collection as known positives (n=356) and archival sera from children which tested negative for Ct antibodies by MIF (n=740). The in-house Pgp3 assay was shown to have greater sensitivity (57.9% overall; 71.5% in women, 39.4% in men) than the other assays, and equal specificity (97.6% compared to 99%, 97.2% and 96% specificity of the three commercial assays). This assay has subsequently been used in several population-based studies in England, looking at the impact of the national chlamydia screening programme [102,144]. The authors have further developed the indirect ELISA to produce a double-antigen “sandwich” ELISA, with greater reported sensitivity (82.9% in women, 54.4% in men) [60].

A separate Pgp3-specific ELISA has been developed and used by Migchelsen and collaborators to measure seroprevalence in a trachoma context [103]. The authors also used the ELISA to determine the seroprevalence of Pgp3-specific antibodies in people of all ages in The Gambia [26]. One complication arising from this study is that, in populations where trachoma and genital Ct infection co-exist, it is not possible to distinguish between Pgp3-specific antibodies that result from ocular or genital infection [26]. A lateral flow assay has also been developed to detect Pgp3-specific antibodies and reports 96% sensitivity and 100% specificity with serum and 81.5% sensitivity and 100%

specificity for whole blood, when compared to the multiplex bead assay previously mentioned [142], although these results are not universal [145].

2.4.5 Novel Antigens

Researchers continue to search for novel biomarkers of chlamydial infection and its scarring sequelae. Transcriptional and proteomic experiments have shown that several chlamydial proteins are differentially expressed and may be of diagnostic value in serology [70,146–148]. Two such proteins, TroA and HtrA, have recently been studied as biomarkers of Ct infection. TroA, encoded by *CT067*, is used in the iron-transport system and is present in infected cells, but less so in EBs [149]. HtrA, encoded by *CT823*, is required for bacterial replication [150] and increases during later developmental stages [151]. These recombinant proteins were used as antigens in ELISAs with sera from women with upper genital tract symptoms, patients from sexually transmitted infection (STI) clinics, blood donors and sexually inexperienced girls with sera being tested for IgG and IgA. IgA was infrequently detected in all patient groups. IgG against both TroA and HtrA was commonly detected in patients with perihepatitis caused by PID and in STI clinic-attendees. Absorbance measured for each antigen was low among blood donors and sexually-inexperienced girls, while absorbance was higher in patients with NAAT-confirmed infection or MIF-positive serology results. The authors suggest that TroA and HtrA antibodies appear following acute Ct infection, with the strongest response detected in upper genital tract infections and when there was evidence of previous or repeated exposure. Differential reactivity to TroA and HtrA, when combined with analyses of the antibodies mentioned above, may be of diagnostic value for reproductive tract complications.

2.4.6 Summary

Many of the assays used in serological surveys are based on the antibody response to one or two antigens, rather than detecting antibodies against a panel of antigens. A recent microarray study showed that in adults with trachomatous scarring, antibodies could be detected against a diverse array of antigens, with eight antigens being associated with scarring [73], three of which were concordant with a prior analysis of the microarray [69]. ELISAs using the three concordant antigens were conducted for further analysis and, in an independent case-control study, two of these were not associated with scarring in adults. Pgp3 was used as a positive control and antibody responses against Pgp3 were strongly correlated between the microarray and ELISA. Recent research in the field of malaria has shown the value of multi-antigen panels, allowing researchers to determine whether or not an individual has been infected [152], how recently [153], and their immunity to re-infection with *Plasmodium falciparum* [154]. Translating this technology to chlamydial antigens may

allow researchers to determine time since infection, number of infections, likelihood of sequelae, or perhaps even if antibodies were due to ocular or genital infection.

2.5 Data analysis and interpretation

Population-based surveys have typically required an estimate of seroprevalence based on the proportion of seropositive and seronegative samples. Few of the current assays present a simple binary response, rather they are based on interpreting continuous quantitative data, and assigning it as positive or negative, based on some established cut-off threshold. It has been shown that the proportion considered seropositive can vary substantially depending on how the threshold is set, particularly if based on reference standards or “known positives” and “known negatives” [103,155]. A simple estimate of seroprevalence may be programmatically practical, but a greater understanding of the (FoI) as a proxy for incidence of infection in a population may be gained from using higher-resolution methods, such as antibody acquisition models; analytical techniques are being explored to measure changes in seroprevalence without setting a threshold or using the quantitative data [156,157].

2.5.1 Methods for determination of thresholds

Thresholds allow the continuous immunofluorescence values produced by an ELISA or MBA to be simplified to a binary or categorical variable. Establishing such thresholds, however, is not straightforward and several methods exist [103]. Mathematical methods can be used to determine threshold values from the distribution of continuous measurements. Internally calibrated approaches (i.e. using only data generated during the study), such as finite mixture modelling [155], expectation-maximisation algorithm [158] or even simply visually determining a threshold between positive and negatives [103] do not require externally calibrated samples- “known positives” and “known negatives” and have been shown to be reproducible and consistent [103,155,159–162]. Migchelsen et al. used four analytical methods to establish a threshold between seropositive and seronegative samples: three were internal reference threshold methods and the fourth was an ROC curve based on externally collected samples which was used to generate three thresholds based on varying levels of sensitivity and specificity [103]. The authors showed that the method selected for threshold specification can greatly affect the population prevalence estimates that are derived, making any comparison between studies more challenging. More traditional threshold methods require external references, making them subject to reference standard misclassification if the reference has been incorrectly classified using infection data [163,164].

2.5.2 Finite Mixture Modelling (FMM)

Techniques such as FMM can be used without reference standards to find a threshold between seropositive and seronegative samples[165]. In its simplest form, FMM sets two Gaussian distributions, a narrow one representing the assumed seronegative samples and a second broader one representing the assumed seropositive samples (Figure 2.6). Although the threshold is commonly defined as the mean of the seronegative samples plus three standard deviations (SD) [155,162,166], this can be made more specific by increasing the threshold to the mean plus four SD [26] or more sensitive by reducing the threshold to the mean plus two SD. Additionally, FMM does not require any reference standards, as the selection of the threshold depends only on the particular samples studied, making it an ideal method for setting a threshold in trachoma-endemic countries, where obtaining appropriate reference samples may be challenging [26,72,103]. Furthermore, this method could be used to identify patients from distinct populations such as ‘never infected’, ‘vaccine-immunised’, and ‘infected’ [159]. FMM has recently been used to identify the number of component titre distributions in patients with laparoscopically-confirmed TFI or controls which was then used to estimate the proportion of TFI attributable to genital Ct infection [167].

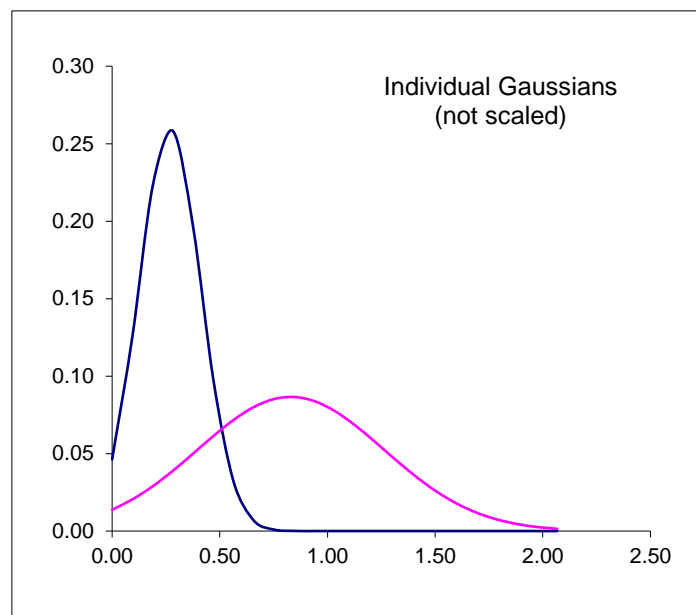


Figure 2.6. Finite mixture modelling composed of two individual Gaussian curves. X-axis measures optical density of ELISA results, while the Y-axis measures proportion of a sampled population. The blue line represents the narrow sub-population of assumed seronegative samples, while the pink line represents the wider sub-population of assumed seropositive samples.

2.5.3 ROC curves

Receiver Operating Characteristics (ROC) curves have commonly been used to set thresholds in serology studies [57,99,112,143,168]. These curves plot assay sensitivity against one minus assay specificity for different threshold values of an assay [169], demonstrating the trade-off between the two characteristics. ROC curves allow for thresholds maximising sensitivity, specificity or Youden's J-index, which balances sensitivity and specificity [170]. When test results with a high value are labelled as positive, increasing the threshold increases specificity whilst decreasing sensitivity, and vice versa for decreasing the threshold. The sensitivity and specificity displayed in the ROC curve rely on the accuracy of the reference assay and standards, highlighting concerns regarding reference standard misclassification [163]. Thresholds for Ct serology in trachoma studies have commonly been set using ROC curves; however, these have been based on a single set of reference standards from Tanzania [99]. The appropriateness of those standards for thresholds for sample sets from other countries appears weak, and potentially underestimates the seroprevalence [100].

2.5.4 Reference standards

Finding an appropriate gold standard test for serology remains a challenge as does finding appropriate reference samples. Samples from patients with NAAT-confirmed infection, have previously been used to define "known positive" samples [57,99,143]; however, infection positivity is not an appropriate reference as patients may not yet have seroconverted or the infection detected was insufficient to induce antibody production [57]. Other serological techniques may also present challenges for confirming positivity. Western blotting and whole cell inclusion immunofluorescence (WIF) have been used to confirm negative samples but use of these techniques assumes their accuracy. WIF detects genus- and species-specific antigens potentially over-estimating the sensitivity of the assay [171], whilst western blots may not be an effective means of detecting antibodies against Pgp3 as antibody recognition is dependent on the native, trimeric conformation of the antibody; the gel electrophoresis step causes the trimeric Pgp3 to denature to a monomeric protein [172]. "Known positives" based on disease status may also be problematic, as some Ct-associated outcomes, particularly those associated with the reproductive tract, are difficult to confirm due to invasive diagnostics [105] or may have more than one aetiology- for example PID may be due to genital infection with either Ct or *Neisseria gonorrhoeae* (NG). [35]. Similarly, serum samples from children or from non-trachoma endemic countries may be inappropriate for an assay designed for use with adults or in another country as non-specific antibodies arising from unrelated, prior infection, may result in different background 'noise' levels [173].

2.5.5 Quantitative antibody levels

New methods are being explored to use the quantitative serology data, rather than simply reducing it to a binary or categorical variable. In low transmission settings, these categorical outcomes may provide lower resolution information than quantitative levels [157]. Robust estimates of Ct transmission are required for the strategic planning, implementation and evaluation of interventions. Quantitative measures of antibodies may be better able to detect changes in high transmission areas and smaller and short-term trends in low-transmission populations [156,166,174].

2.5.6 Summary of analysis and interpretation of serological assays

The current methods of determining known references are less than ideal, reflecting the challenges in selecting a 'gold standard' reference. Clinical signs and infection status are inappropriate reference standards and comparator serological assays must be carefully selected, taking into account their known limitations, which may exclude their use as gold standards for determining thresholds. Internally calibrated methods remove the need to use reference standards, and set thresholds specific to the samples being tested, eliminating the need to source standards specific to the population being studied. Finally, more robust analysis can be performed by using the quantitative data, providing a sensitive measure of transmission changes.

2.6 Serology used in population-based surveys

The following section highlights some of the population-based studies using serological techniques to measure antibodies to Ct. Monitoring the prevalence of anti-Ct antibodies over time has strong potential as a surveillance tool in post-elimination settings as well as a tool to monitor and evaluate programme effectiveness [27,97,98]. It may also provide an understanding of what additional methods must be undertaken in regions where trachoma has not successfully been eliminated, despite MDA and other interventions [175]. Population-based serology studies are being explored as an alternative measure of prevalence of genital Ct infection, by providing a measure of age-specific cumulative incidence. Due to the often asymptomatic nature of urogenital chlamydia, ascertaining the prevalence of infection depends on the population tested [176]. Surveillance is commonly based on case reporting alone with no reporting on numbers of patients tested. Even when a testing denominator is known, positivity does not equal prevalence because the population tested has a different inherent risk than the general population [177].

In the first half of this section, we track the use of serology as a potential monitoring tool for trachoma programmes, whilst in the second half, we highlight serology's use in population-based studies of genital Ct infection.

A systematic literature search of online databases (PubMed, Cochrane, Lilacs, Scielo, Scopus and Web of Science) was conducted in June 2016, and updated in April 2018, following PRISMA guidelines [178] (Figure 2.7). The literature search used the following keywords: trachoma; *Chlamydia trachomatis*; chlamydia; prevalence; serology; epidemiology; diagnostics; genital; and ocular. The search was limited to peer-reviewed *Chlamydia trachomatis* serology papers published in English, French, Spanish and Dutch. The reference lists of retrieved articles were hand-searched to identify other studies that may have qualified for inclusion in the review. Studies were eligible for inclusion if they used population-based sampling, including if the participants were drawn from a sub-national geographic region of the country. No age limits were set on the population, as trachoma studies most commonly involve children, while genital chlamydia studies typically involve teenagers and adults. Participants must have provided a blood sample for analysis. No intervention or comparison groups were relevant to the inclusion criteria in this review. The outcome was the prevalence of antibodies against Ct, defined as the positive result of a serological assay by the study investigators. Studies were excluded if they were case-control studies or if samples were collected from patients at an STI or sexual health clinic or ophthalmology clinic (Figure 2.7)

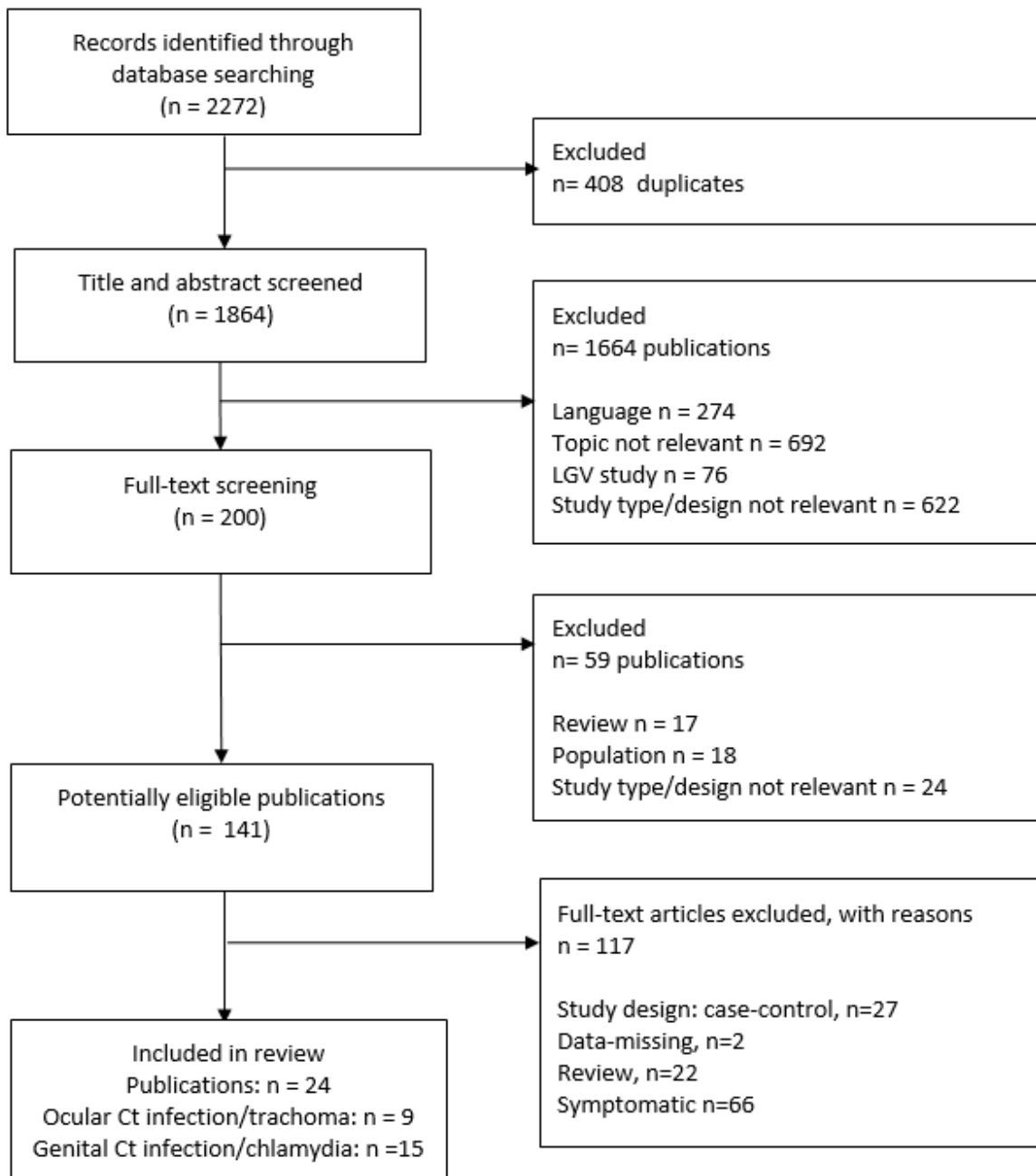


Figure 2.7. Flow chart of publications identified and excluded for this review.

2.6.1 Ocular Ct infection

The following studies show the advance of serological techniques and their use from a simple measure of seroprevalence to proxy indicators of the transmission and FoI. We have included the demographic information and seroprevalence of each study in Table 2.1.

Table 2.1. Summary of ocular Ct studies

Study	Location	Population (n)	Sample	Assay	Antigen	Immunoglobulin	Reference standards	Sensitivity (95% CI)	Specificity (95% CI)	Seropositivity (95% CI)
Goodhew et al., 2012 [99]	Tanzania	Children aged 1-9 years old from 4 villages, post MDA (3 rounds) (n=160)	dried blood spots (DBS)	in-house multiplex bead assay (MBA)	Pgp3	IgG	Ct-infection PCR-positive samples from Tanzanian children (n=11); assumed-negative serum samples from US children (n=122)	90.9% (62.2-98.4); 10 of the 11 PCR positive DBS were also considered positive by MBA	97.5% (93.0-99.2); three assumed-negative samples were positive for antibodies against Pgp3	49.4% (41.7-57.1)
					CT694			90.9% (62.2-98.4); as above	98.4% (94.2-99.6); two assumed-negative samples were positive for antibodies against CT694	46.3% (38.7-54.0)
Goodhew et al., 2014 [64]	Tanzania	Children aged 1-6 years old, baseline and	DBS; swabs collected	MBA	Pgp3	IgG-baseline	As in [99]	96.1% (87.0-98.9); 49/51 PCR-positive	not possible to calculate from the	63.9% (57.2-70.2) for any IgG response

		after one round MDA (n=208 tested for IgG at baseline; n=184 tested for IgA)	for PCR test of infection			IgG-post MDA		samples tested positive for IgA antibody against Pgp3	data provided	no seroreversion
						IgA- baseline	Ct-infection PCR-positive samples from Tanzanian children (n=10); assumed-negative serum samples from US children (n=122)	96.0% (86.5-98.9); 48/50 PCR-positive samples tested positive for IgA antibody against Pgp3		53.2% (46.6-60.1) for any IgA response
					IgA- post MDA	no seroreversion				
					CT694	IgG-baseline	As in [99]	96.1% (87.0-98.9); 49/51 PCR-positive samples tested positive for IgA antibody against CT694		no seroreversion
					IgG-post MDA	no seroreversion				
						IgA- baseline	As for Pgp3-specific IgA	92.0% (81.2-97.0); 46/50 PCR-positive samples tested positive for IgA		no seroreversion
					IgA- post MDA	no seroreversion				

								antibody against CT694		
Martin et al., 2015 [66]	Tanzania	Children aged 1-6 years old from one village in a hyperendemic setting (n=208); children aged 1-9 years old in 8 villages in a mesoendemic setting (n=987) and hypoendemic setting (n=680)	DBS; swabs collected for PCR test of infection	MBA	Pgp3	IgG	As in [99]	Not described	Not described	Hyperendemic: 62.0% (55.4- 68.6); Mesoendemic: 32.7% (30.0- 36.0) Hypoendemic: 21.2% (17.7- 24.6)
					CT694					Hyperendemic: 61.5% (54.9- 68.1); Mesoendemic: 34.0% (31.0- 37.0) Hypoendemic: 18.4% (15.1- 21.7)
Martin et al., 2015 [27]	Tanzania	Participants of all ages in a community where trachoma was eliminated in 2005 (n=571)	DBS; swabs collected for PCR test of infection	MBA	Pgp3	IgG	As in [99]	Not described	Not described	33.8% (30.0- 37.8) seropositive against at least one antigen
					CT694					

Pant et al., 2016 [100]	Nepal	Children aged 1-9 years old from 3 post-MDA communities (n=68)	DBS; swabs collected for PCR test of infection	MBA	Pgp3	IgG	As in [99]	Not described	Not described	1.50% (0.04-7.9)
					CT694					0% (0-5.3)
Zambrano et al., 2016 [98]	Nepal	Children aged 1-4 and 9 years from 15 clusters across two districts (n=794)	DBS; swabs collected for PCR test of infection	MBA	Pgp3	IgG	As in [99]	Not described	Not described	2.40% (1.5-3.7)
Cocks et al., 2016 [72]	Fiji	Children aged 1-14 years in 30 randomly-selected villages in Western District (n=593)	DBS; test for antibodies against Ct and <i>Treponema pallidum</i>	ELISA	Pgp3	IgG	Threshold set using finite mixture modelling; no reference standards required	Not described	Not described	20.9% 17.8-24.6)
West et al., 2018 [65]	Kongwa, Tanzania	Children aged 1-9 years in 52 communities enrolled in a clinical trial of surveillance studies; MDA	DBS; swabs collected for PCR test of infection	MBA	Pgp3	IgG- baseline	As in [99]	As in [99]	As in [99]	31.1% (29.1-33.1)
						IgG- one year follow-up				36.8% (34.8-38.9)

		completed 2013 (n=2111)								
Butcher et al., 2018 [189]	Temotu and Rennell & Bellona, Solomon Islands	Participants of all ages in villages that had received MDA six months prior (n=1,511); DBS collected from 1,499 participants	DBS; swabs collected for ddPCR test of infection	ELISA	Pgp3	IgG	Threshold set using finite mixture modelling; no reference standards required	Not described	Not described	42.2% (39.8- 44.8)

The first modern study by Goodhew et al., from Tanzania, described the prevalence of antibodies against Pgp3 and CT694 in dried blood spots (DBS) collected from children aged 1-9 years (n=160) from four villages that had received three rounds of azithromycin as part of an MDA campaign [99] (Table 2.1). Clinical signs were recorded, and conjunctival swabs were collected to test for infection using PCR. The level of antibodies against Pgp3 and CT694 were measured using an in-house MBA. The thresholds for seropositivity were set using ROC curves, determined using delinked serum samples from children from the United States as negative controls and dried blood spots from Ct-infection PCR positive children from Tanzania as positive controls. Samples from Haitian children were also included as negative controls. The median fluorescence intensity minus background (MFI-Bkgd) values for Pgp3- and CT694-antibodies measured in Tanzanian samples were higher than those from both the Haitian and American samples. The seroprevalence of antibodies against both Pgp3 and CT694 was higher than the prevalence of either clinical signs or PCR positivity, with prevalence of antibodies against both antigens increasing with age. Older children were more likely to be seropositive but infection-negative and clinically-negative, suggesting past exposure with antibodies persisting after infection had been cleared, whilst most children under 3 years of age who were seropositive were also infection-positive or had clinical signs of trachoma. Six children who had signs of trachoma failed to exhibit an antibody response to either Pgp3 or CT694, which may suggest that these children failed to develop an immune response to Ct infection or that the assay is not as sensitive as stated, when compared to clinical signs; it may also suggest that the grader mis-graded TF in these children, or that the follicles were of a different aetiology. At the community-level, villages with higher prevalence of trachoma also had higher prevalence of seropositivity for both Pgp3 and CT694.

The second study, also from Tanzania and by Goodhew and colleagues, examined the change in antibody levels from baseline to six months after a round of MDA [64] (Table 2.1). Children aged 1-6 years old (n=173) provided DBS at both baseline and 6 months post-MDA, in addition to undergoing a clinical examination and providing an eye swab for infection testing. Both IgG and IgA against Pgp3 and CT694 were measured, as in [99]. At baseline, 47% of children were clinically positive for TF/TI, 25% of children were infection-positive, IgG seroprevalence was 64% and IgA seroprevalence was 53%. Both IgG and IgA against Ct-antigens increased with age. Data showed that although the levels of immunoglobulin (both IgG and IgA) decreased post-MDA, in no case did antibody levels decrease to a level where the sample would be considered negative. The mean decline in IgA antibody levels for antibody-positive children was greatest in the youngest children for both Pgp3 and CT694. No specific data were presented for IgG levels declining. The authors summarised that IgG may decline less significantly due to repeated Ct exposures, which induce memory B cells or plasma cells, as part

of the humoral immune response [179] whereas IgA immune response are shorter-lived particularly after mucosal infection with other organisms [79]. This would suggest that IgA is not a suitable target antibody for detection as it has a shorter duration than IgG. All IgA-positive samples were also IgG-positive. However, of the samples positive for IgG against either antigen, 21% were seronegative for IgA against both antigens, 19% were seronegative for anti-Pgp3 IgA and 33% were seronegative for anti-CT694 IgA.

An additional study, by Martin et al., in the same region of Tanzania used serological data from hyper-, meso-, and hypoendemic settings [180] to calculate the basic reproduction rate (R_0) for each level of endemicity [66] (Table 2.2). Dried blood spots were collected from children aged 1-9 years ($n=1208$) and tested for antibodies against Pgp3 and CT694 using MBA. The R_0 was calculated for each antigen in each endemicity assuming a constant (Foi) over age, which the authors caution may only hold true for young children, rather than the whole population (Table 2.1). When compared to R_0 values calculated using maximum likelihood estimation [31], the values presented in Table 2.2 are much higher.

Table 2.2. Calculated R_0 in communities with three levels of endemicity in Tanzania, including 95% confidence interval (95% CI). Modified from [66].

	R_0 Pgp3 (95%CI)	R_0 CT694 (95% CI)
Hyperendemic	29.4 (21.1-37.7)	28.3 (19.9-36.8)
Mesoendemic	8.1 (6.3-10.0)	7.8 (6.9-9.6)
Hypoendemic	2.8 (2.1-3.6)	2.8 (1.6-4.0)

Age-specific seroprevalence appears to be a reasonable proxy for disease in determining transmission rates for trachoma in these communities. The higher R_0 values seen in hyperendemic communities reflect a greater slope of the age seroprevalence curve and the prevalence of clinical signs. This study is a useful proof-of-concept that age-specific seroprevalence levels can be used as a proxy measure of the transmission of ocular Ct infection; it may be possible to use serology as an indicator of exposure. The authors caution that further studies are needed at district level, which is the functional unit for programmatic purposes, as well as studies with larger sample sizes, particularly for children at the youngest ages.

A third Tanzanian study [27] took place in a community where ocular chlamydial infection was eliminated with MDA of azithromycin between 2000 and 2002; elimination was confirmed in 2005 [181] (Table 2.1). The authors highlighted some of the challenges associated with ‘traditional’

trachoma survey including inter-observer standardisation [16,180,182] and the likelihood that follicular conjunctivitis may be caused by non-chlamydial bacteria, particularly in post-MDA settings [183]. In this study, the authors use the age-specific seroprevalence of antibodies against Pgp3 and CT694 to model the FoI before and after MDA. All residents were invited to undergo eye examinations and had an ocular swab collected for PCR infection testing. DBS were collected from all participants (n=571), which were then tested for antibodies against Pgp3 and CT694 using the MBA. The overall prevalence of TF/TI was 4.6% (6.5% in 1-9-year olds) whilst 21.5% of the participants had signs of scarring trachoma. Seropositivity increased with age, such that by 40 years of age over 90% of participants were positive for antibodies to at least one antigen and 60% were positive for antibodies to both. Of 200 participants aged 1-9 years, only 3.5% had positive responses to one antigen. No infection was detected. Seroconversion rates (SCR) were modelled using a simple reversible catalytic model using maximum likelihood methods and the best fit was provided with a change in the FoI 10-15 years prior, which is consistent with MDA having occurred between 2000 and 2002. The authors note that children born post-MDA have virtually no antibody response to Pgp3 or CT694, strongly suggesting a lack of Ct transmission, supported by the lack of clinical signs and infection. With regards to the few young children who have detectable levels of antibodies (3.5%), the authors suggest that these may be children who have acquired ocular or respiratory Ct-infection at birth from a mother with genital Ct infection but also that the previously determined specificity of the assay is 96-98% and thus these may be false positives. The authors acknowledged that their study represents only one community with a small sample size and suggest that additional studies be done in other post-MDA regions before their findings can be generalised. An additional limitation of the study was the lack of pre-MDA serology data. These data would assist in determining antibody longevity and how titres change due to repeated exposure. However, as the scale of trachoma-intervention programmes has rapidly increased in recent years, few endemic-populations are likely to have collected pre-MDA serological data; this should be considered for future intervention activities.

A trachoma study by Pant and collaborators examined the seroprevalence of anti-Ct antibodies from 68 children aged 1-9 years in three post-MDA communities in Nepal [100] (Table 2.1). Following three rounds of MDA in 2008-2010, the prevalence of TF remained relatively high in Achham district, and an additional round of MDA was carried out in 2014. The three villages were selected on their accessibility and convenience. In these three communities, participating children had their eyes examined for clinical signs of trachoma, an ocular swab was collected for NAAT and DBS collected. No clinical disease was detected, nor was any infection detected using PCR. Only one sample tested

positive for antibodies against Pgp3; none were positive for antibodies against CT694. Although it would not be surprising to see a low level of prevalence in these three communities, knowing that the prevalence of TF was above 5% as recently as two years before the study, and that serology measures cumulative exposure to Ct infection, it seems that unlikely that only 1 child would have antibodies against Ct. No mention is made of the specificity of the assay. One possible explanation for this possible under-estimation of seroprevalence may be the threshold set for seropositivity. This threshold was set against 'negative' American serum samples and 'positive' Tanzanian DBS (outlined in [99]). If the baseline antibody level of the Tanzanian samples is greater than the baseline level of Nepalese samples – as seen in the original MBA study looking at data from Haiti and the USA – the Tanzanian threshold may not be appropriate for the Achham samples and positive samples may have been misclassified [184], highlighting the need for population-specific thresholds. The authors noted that the sample size is very small and thus the results should not be generalised but failed to address any concerns about sensitivity or specificity.

A second, larger study [98] was conducted in Nepal by Zambrano and colleagues who looked at the prevalence of TF, infection and anti-Ct antibodies in children. They used the new WHO guidelines which recommend a population-based prevalence survey at least 2 years post-MDA measuring both TF and TT [185] (Table 2.1). The study took place in two districts which were at least two years post-MDA. In low prevalence settings, the specificity of TF diagnosis is decreased, due to potential misgrading and the fact that follicular disease may have different aetiology [98]. It is thought that additional data, for example a test for infection or a test for antibodies against Ct, may provide additional evidence of elimination [185]. A random sampling of 15 clusters in two districts was selected in which 50 children aged 1 – 9 years had their eyes examined (n=2021). Selected children aged 1 – 4 and 9 years (n=794) had ocular swabs collected for infection testing and DBS collected for anti-Pgp3 IgG testing using the in-house MBA [99]. The prevalence of TF in surveyed children in both districts was $\leq 0.2\%$. Only one case of infection was detected. The data from this study suggest there was no ongoing transmission of ocular Ct infection in the two districts, with the low age-specific distribution suggesting the absence of exposure to Ct. The prevalence of anti-Ct antibodies was $< 5\%$ and is similar to levels seen in a Tanzanian community where there was no infection and only low levels of trachoma (6.5% in 1-9 year olds) [27]. This study adds to the evidence for using serology as additional evidence of elimination.

A recent study in the South Pacific by Cocks and colleagues showed the potential for integrating surveys for NTDs; in particular yaws and trachoma, both of which are endemic in the region [72] (Table 2.1). A population-based survey was conducted in the Western Division of Fiji to measure the prevalence of yaws (infection with *Treponema pallidum* subsp. *pertenue*) and trachoma. A total of 30 villages were selected randomly based on probability proportional to size, then 30 households in each village were selected. All children aged 1 – 14 years (n=607) in the selected households were invited to participate. Each child was examined for signs of yaws, and 593 children had a DBS collected to test for antibodies against *T. pallidum* and Ct. Samples were tested using the *T. pallidum* particle agglutination test and a Pgp3 ELISA [103] to detect antibodies against *T. pallidum* and Ct, respectively. No children were found to have yaws, nor were any children positive for antibodies against *T. pallidum*. Although children were not examined for signs of trachoma in this survey, a previous study found there to be a low prevalence (2.8%) of active trachoma in Western Division [186]. The current study found a seroprevalence of 20.9% in surveyed children. There was only a moderate increase in seropositivity with age, and many 1-year old children were already seropositive, suggesting that seroprevalence may reflect transmission in the birth canal. In such a situation, it would be useful to compare serological results with the prevalence of genital Ct infection as it has been previously noted that the prevalence of genital Ct-infections is high in Fiji [187]. This study shows that trachoma and yaws are not significant health problems in the region studied, though further studies are required to determine the prevalence of genital chlamydia.

To measure the longevity of the immune response, West et al. collected DBS from children aged 1 – 9 years in 52 communities in the Kongwa district of Tanzania [65] at two time points (Table 2.1). Kongwa was previously considered hyperendemic for trachoma, but following MDA, the prevalence of TF was <10% in 2013 [188]. At baseline, participating children had their eyes examined for signs of trachoma, swabs collected for infection testing and a DBS collected for antibody testing. One year later, additional DBS were collected; a total of 2,111 children had DBS at both time points. At baseline, the prevalence of TF was 5.2%, the prevalence of ocular Ct infection was 4.6% and 31.1% of children had antibodies against Pgp3, as measured using MBA. One year later, seroprevalence had increased to 36.8%. Of the children positive at baseline, 42 seroreverted to negative after one year, while of those negative at baseline, 143 seroconverted to positive. At baseline, the seroreverters had lower average MFI-Bkgd measurements than those who were remained positive at follow-up; at follow-up the seroreverters had significantly higher MFI-Bkgd measurements than those who were seronegative at both time points. Those who were seronegative at both time points were more likely to be 1- 3 years old, while those who were seropositive at both time points were more likely to be

aged 7-9 years, suggesting some stability in seropositivity for at least one year in low-prevalence communities. The authors suggested that the lack of age-specific increase in the prevalence of antibodies that is typically seen in low-endemic areas may be partially due to seroreversion rather than a lack of infection.

Serology has been used in combination with ocular examination, photographic assessment and droplet digital PCR to re-assess evidence of on-going transmission of ocular Ct in two provinces (Temotu and Rennell & Bellona) in the Solomon Islands [189] (Table 2.1). An initial population-based prevalence survey found the prevalence of TF to be 26.1% in children 1-9 years old, although the prevalence of ocular Ct infection was very low (1.3% in 1-9 year olds) as was TI (0.2% in 1-9 year olds) and TT (0.1% in participants ≥ 15 years old) [190]; this was dissimilar what was seen in Kiribati, another South Pacific nation considered endemic for trachoma [191], and led researchers to question the underlying biology of TF in the Solomon Islands. Villages which had undergone mapping at baseline were e-surveyed: participants of all ages underwent ocular examination, had photographs taken of the everted right tarsal conjunctiva and dried blood spots were collected. Children aged 1-9 years had swabs taken of the everted right tarsal. Six months post-MDA, the prevalence of TF was 14.2%, the prevalence of infection remained low (1.8%), both in 1-9-year-old children and seroprevalence was 42.2% overall. Upon further analysis, ELISA results showed that an equal proportion (~80%) of TF-positive and TF-negative children lacked antibodies against Pgp3, unlike in other countries, where greater seropositivity was seen in TF-positive children, suggesting that trachoma was likely not the cause of the follicles. Additionally, the greatest increase in seroprevalence was seen in 16-25-year olds, suggesting sexually-acquired genital Ct infections. The few cases of conjunctival scarring detected in children were not typical of trachomatous scarring and were detected in children without any antibodies against Pgp3. The combined results of this study suggest that while there may be some Ct infection, likely both genital chlamydia and a small amount of ocular chlamydia, most of the cases of TF are likely due to an infection other than Ct. This study assessed age-specific infection prevalence, seroprevalence and clinical results together across the full age range of the population to provide further clarity as to what may be causing TF in this population.

Summary of Seroprevalence Studies for Ocular Chlamydia

Although only a handful of trachoma serology studies have been published thus far, there is clear development of the technique and its potential to measure the impact of elimination programmes. The majority of studies published to-date has been conducted using an in-house MBA. Use of a MBA

allows the assay to be expanded to include antigens from numerous infections and diseases, which could be used to monitor seroprevalence across programmes [99]. However, it has been noted that the cost of such technology may prevent it from being adopted in trachoma-endemic countries [97]. Enzyme-linked immunoassays (ELISA) or lateral flow assays may offer a more practical solution, and feasibility is currently being explored [26,103,142,145]. The manner in which an appropriate threshold is set may also present a problem and is not exclusive to trachoma serology. There is a lack of an appropriate reference standard and experts have yet to agree as to what samples may constitute a true positive or a true negative reference, with regards to infection and disease history. Trachoma serological studies to-date have been led by the Centres for Disease Control and Prevention (CDC) and have predominantly used samples from Tanzanian populations. The original study shows that the measured median antibody levels can vary between populations, particularly between endemic and non-endemic populations. This should be taken into account when assays are used to measure seroprevalence in other populations as thresholds set for one population may over- or under-estimate the seroprevalence in different populations. Population-specific thresholds should be set using in-country samples rather than relying on non-endemic samples.

2.6.2 Genital Ct infection

In the section below, we have chosen to focus on the unique features of each study, which highlight the development of serology and its interpretation in studies focusing on genital chlamydia infection. We have included the demographic information and seroprevalence of each study in Table 2.3.

Early chlamydial serological studies were primarily concerned with determining the rate of exposure to 'chlamydial agents' [88]. Early diagnostic studies used cell-culturing systems [192], which required significant technical skill and expertise. The development of a MIF assay with whole EBs as antigen [44,193] was lauded as being sensitive, simple and much faster than traditional culture methods. It was used by Treharne and colleagues in a survey of serum samples from healthy blood donors from London (UK) which were screened for anti-chlamydial IgG and IgM [88] (Table 2.3). The MIF previously developed [39,194] divided 'subgroup A Chlamydia' into 14 subgroups. The modified protocol measured antibodies against four subgroups of chlamydia: serotypes responsible for i) hyperendemic trachoma, ii) "paratrachoma" [infection with *C. pneumoniae*], iii) LGV, and iv) human chlamydia infections "arising from birds or other animals" [44] [infection with *C. psittaci*]. The authors suggest that MIF positivity in women may correspond better with cell culturing than in men; however, the authors rightly pointed out the insensitivity of cell culture systems used in isolating chlamydia.

In a retrospective study in Helsinki, Puolakkainen et al. [89] used CF to screen serum samples from 60,000 patients of all ages with suspected viral infections for antibodies against primarily viral antigens, but also included group-specific CF antigen from Ct, serovar D (Table 2.3). It is important to note that the CF test detects antibodies against both *C. psittaci* and Cp as well as *C. trachomatis*. The authors also noted that the sensitivity of the CF assay is very much dependent on antibody titre, such that Ct infections seldom elicit a sufficiently strong antigenic response. The authors concluded that many of the seropositive patients had symptoms matching psittacosis, rather than genital chlamydia.

A study by Jonsson and colleagues in Sweden was conducted to assess the prevalence of Ct using culture and serology and their relationship with possible risk factors [90] (Table 2.3). The population-based study included the complete 19-, 21-, 23- and 25-year old cohorts of women registered in the

catchment area of Ålidhem health centre in Sweden. Participating women (n=529) completed a self-administered questionnaire and had cervical and urethral specimens collected for culturing. Cultures were considered positive if cytoplasmic inclusion bodies were detected. Sera were collected from participants and MIF was used to detect antibodies against Cp, *C. psittaci*, and Ct. Only 15 women (2.8%) were culture-positive for Ct-infection, nine (60%) were also seropositive for antibodies against Ct. Overall, 130 women (24.6%) were seropositive for IgG against Ct. Antibodies against Cp were detected in 207 women (39.1%). There was no statistically significant difference in the prevalence of women with antibodies against both Ct and Cp compared to those with antibodies against only Ct ($X^2=2.65$, p-value=0.1034). Of those women who were aware of a previous Ct infection, 60% were seropositive, compared to 17% seropositivity in women who were unaware of previous Ct infection. Commenting on the fact that only 60% of women were aware of a previous infection or a current infection, the authors stated that the exact conditions required for seroconversion following infection are unknown; nor is it known how long a detectable antibody response persists. The authors also commented on the specificity of MIF, having tested samples against three *Chlamydia* species. The authors felt that MIF was able to distinguish between antibodies against Ct and Cp without cross-reacting. The authors also found that reported PID was correlated with Ct-seropositivity. These findings agree with other studies [195–199] and suggest a possible role for Ct antibodies in determining the aetiology of female infertility.

As part of a study into the prevalence of sexually transmitted diseases in Tamil Nadu, India, a population-based, probability proportional to size cluster survey was undertaken [200] (Table 2.3). Households in three districts were selected, with adults, both men and women, between 15-45 years invited to participate. Urine and blood samples were collected for PCR and IgM ELISA testing, respectively. A total of 1,849 samples were collected: 1,066 from females and 783 males. Sera were tested using a commercial IgM ELISA [201] with an overall seroprevalence of 2.4%. The seroprevalence in women was significantly higher (3.3%) than seroprevalence in men (1.3%) (X^2 , $p<0.05$). PCR positivity was lower, with only 16 of the 1,444 urine samples testing positive. The authors did not compare the proportion of PCR-positives to those that are seropositive, stating only that for participants in which results from the two tests are not concordant, this may be because infection have been resolved in a 'substantial portion' of IgM positive cases, or that infection had ascended to the upper genital tract. However, IgM is not a suitable marker for infection as the conditions under which seroconversion occurs or for how long IgM antibodies persist remain unclear.

A simple seroprevalence study was undertaken by Satpathy et al. to determine the prevalence of serovar-specific antibodies against *Chlamydia* in healthy male blood donors (n=844) in Delhi, India [92] (Table 2.3). Sera from 844 male blood donors were assayed using MIF to detect antibodies against pooled EBs from Ct (serovars A-C, D-K, L1-L3), *C. psittaci* and Cp. The authors state that antibodies against Ct, Cp, *C. psittaci* and *C. pecorum* are highly cross-reactive, with 47% of samples positive for antibodies against Ct serotypes A-C cross-reacting, 14% of samples positive for antibodies against Cp cross-reacting and 33% of samples positive for antibodies against *C. psittaci* cross-reacting. With many adolescents being infected with Cp as respiratory infections, screening of normal sera showed high positivity for antibodies against *Chlamydia* species, especially Cp [56]. The authors admitted that the high level of cross-reactivity between serovars and species made interpreting results for individual antigen groups challenging.

In Denmark, women requesting abortion are tested for the presence of Ct and treated if positive. Baczynska and collaborators took the opportunity to test women requesting abortion to determine the prevalence of *Mycoplasma genitalium* (MG), *M. hominis* and Ct using culture and PCR, as well as the prevalence of antibodies against these bacteria using a MOMP-specific commercial ELISA (Medac) [93] (Table 2.3). Endocervical swabs were collected for culturing *M. hominis*, and PCR detection of *M. hominis* [202], *M. genitalium* [203] and Ct. The authors compared the prevalence of Ct infection with the historical prevalence of infection (8-10%) [204] and noted that the apparent increase in prevalence may have been due to a true rise in prevalence or the use of improved diagnostic techniques. There was poor correlation between the detection of bacteria and the presence of specific antibodies. The authors suggested that PCR-positive/serology-negative women may have been sampled before IgG antibodies could be produced, whilst PCR-negative/serology-positive women are likely to have experienced previous infection.

To establish the prevalence of anti-Ct IgM and IgA in pregnant women in Venezuela, De Freitas and colleagues collected serum samples from 84 pregnant women, aged 14-43 years, attending University Hospital “Antonio Patricio de Alcalá” in Cumaná, Estado Sucre [94] (Table 2.3). A commercial ELISA assay (Diagnostic Automation INC) was used to detect IgA and IgM antibodies against a proprietary “LGV type 2 broadly reacting antigen” [205]. The authors stated that IgM is indicative of active, current infection, whilst IgA is indicative of early infection or secondary

infection. However, no samples were collected for culturing or NAAT, to confirm infection. Most patients, including those with either IgM or IgA, were asymptomatic. The authors found the prevalence of both antibodies to be greater in younger women (≤ 24 years of age).

A retrospective population-based cross-sectional survey was conducted in England by Horner et al., in which 4,732 serum samples were assayed with an in-house IgG-specific Pgp3 ELISA [102] (Table 2.3). The purpose of the study was to determine how seroprevalence of anti-Pgp3 antibodies changed over time and with age. Unlinked residual sera submitted to laboratories in England for routine microbiological or biochemical investigations were tested; sera known to have been collected from GUM clinics were excluded. Sera were limited to those collected between 1993 and 2010 from women aged 17-24 years. Samples were tested for anti-Pgp3 IgG. The overall seroprevalence was 17.5%. Seroprevalence increased with age and with time, between 1993 and 2002. In more recent years, age-standardised seroprevalence decreased in 17-22 year olds although this was only statistically significant in 20-22 year olds; there was no consistent trend in seroprevalence for 23-24 years olds in this time period. This may suggest that exposure to antibody-inducing infection may have declined in the latter years, potentially in response to public health activities promoting increased screening for Ct [206] and safer sexual health practices [207].

In response to rising rates of reported genital Ct infection in the Netherlands, van Aar and colleagues explored the prevalence of IgG against Ct in the general population in 1996 and 2007 using samples from large population-based serum banks (Table 2.3). This study covered a time when enhanced efforts were undertaken to reduce the prevalence of genital infection [95]. Samples were used from cross-sectional population-based serosurveillance and were tested for IgG against a synthetic peptide from the immunodominant region of MOMP using a commercial ELISA (Medac). When compared to MIF, the sensitivity and specificity were 71.4% and 97.3%, respectively. A total of 3,716 samples were tested: 1,579 from 1996 and 1,597 from 2007. In women, the seroprevalence increased with age, but not in men. In the younger women (15–24 years), the seroprevalence increased over time; for the older age group (25–39 years), the seroprevalence decreased. The authors noted the difficulty in interpreting results over time, as it is not possible to determine the precise time span between often-asymptomatic Ct infection and testing. Based on their findings, the authors hypothesised that anti-Ct antibodies may persist longer in women than in men, which may be due to a stronger immune response in the upper genital tract, in agreement with previous results

from Geisler et al. [208].

Ishak et al. undertook a retrospective study of serum samples from 1710 individuals in Brazil to determine the seroprevalence of antibodies against Ct [96] (Table 2.3). Samples were randomly selected from previous investigations at the Virus Laboratory of the Universidade Federal do Pará, which was established to determine the frequency of infectious agents in the Brazilian population. The samples had been collected from both urban and rural populations, including patients with respiratory illnesses, myocardial infarctions, villagers of Afro-descendant communities and indigenous groups; no further demographic information such as age or gender was included. A commercial indirect immunofluorescence assay (IIF; BioMerieux) was used to detect IgG and IgM against Ct serotype L₂. Positive IIF samples were then tested for specific IgG at a dilution of 1:512. Samples positive for IgG at 1:512 were tested for specific IgM. A portion of the samples was tested with MIF against Cp and the serovars of Ct, including A, B, Ba, and C (associated with ocular Ct infection); D, E, F, G, H, I, J and K (associated with genital infection); and L1, L2 and L3 (associated with lymphogranuloma venereum [67]). Samples that tested positive against more than one serovar were serially titrated as the serovar with the highest titre is assumed to have caused the most recent infection. The authors believed the prevalence of antibodies against serotype A (15/237, 6.3%), a strain associated with trachoma, is an indication of endemic trachoma in the Amazon region of Brazil, dating back several centuries [209] and that trachoma has spread to urban and other non-urban populations, despite a lack of patients with conjunctivitis or any other ocular disease resembling trachoma.

Woodhall and colleagues used anonymised sera from a nationally representative health survey to measure the impact of a national programme of opportunistic screening for Ct in England [59] (Table 2.3). Serum samples were collected as part of the Health Survey for England [22], which is a nationally representative health and lifestyle survey. Sera from participants aged 16-44 were tested using two in-house assays—an indirect ELISA and a double antigen “sandwich” ELISA to detect Pgp3-specific IgG [57]. They found lower seroprevalence in men possibly due to lower sensitivity, as previously noted in [57]. Seroprevalence increased with age, peaking in 30-34 year old women and in 35-39 year old men. These results may be comparable to those in [60], as both studies used the same in-house Pgp3-specific ELISA; however, the populations represented in each study are different.

Using stored sera from a New Zealand birth cohort, an in-house double antigen (sandwich) Pgp3-specific ELISA was used by Horner and collaborators to measure IgG levels in participants at ages 26, 32 and 38 years [60] (Table 2.3). The authors developed a double-antigen ELISA and measured the sensitivity and specificity against a previously used indirect ELISA [57], with sensitivity being defined as the proportion of individuals with past infections identified as positive by the assay [210]. The sensitivity was found to be 82.9% in samples from women who were NAAT-positive for Ct-infection at least one month before sample collection and 42.6% in men. Specificity was determined using NAAT-negative paediatric sera and was 97.8%. The assay was used to test stored serum samples from a New Zealand birth cohort study that had also collected information on self-reported Ct infection and sexual behaviour [211]. The double-antigen ELISA identified 1.75 times more the number of self-reported Ct cases than the previous indirect ELISA. IgG against Pgp3 persisted in most men (83.8%) and women (96.5%) who did not report a subsequent Ct diagnosis. The authors highlight the relatively high seroprevalence in women and men who did not report ever having been diagnosed with Ct infection, indicating that many infections were either undiagnosed or unreported. These samples were re-analysed using a Pgp3-specific double-antigen sandwich ELISA by Righarts et al. [212], with increased sensitivity compared to the indirect ELISA [60] (Table 2.3). In both women and men, incidence rates increased up to age 26 years, then decreased; seroprevalence increased with age. Incidence of chlamydia, measured either through self-reported diagnosis or serology, increased with number of sexual partners. In multivariate analysis, higher education was protective in women, while same-sex contact was found to be protective in males. This analysis showed that by age 38 years, 38.1% of women and 16.7% of men had ever self-reported a chlamydia diagnosis. Of these self-reported diagnoses, 80.0% of women and 29.0% of men had seroconverted by this age, which may suggest that males are less likely to mount an immunological response to Ct infection and also support the noted gender differences in the sensitivity of the assay as previously identified in [60].

MIF serology has been used to characterise past exposure to genital Ct infection and its association with developing uterine fibroids [213] (Table 2.3). Ct infection can stimulate an inflammatory immune response, which may lead to tissue repair and regeneration potentially causing uterine fibrosis [214,215]. African-American women participating in a prospective cohort study of fibroid development underwent transvaginal ultrasound and provided self-reported data and serum samples. Serum samples were tested using MIF for antibodies against whole EBs; researchers found seropositivity of 57%. In this study, seropositive women were more likely to be less educated,

parous, more likely to have ever smoked, to be heavier alcohol drinkers, to have an increased number of sexual partners, to have been younger at first sexual encounter and to have used depot medroxyprogesterone acetate (Depo-provera) as a method of birth control. Both primary (odds ratio=0.68) and multivariate analysis, adjusting for age (adjusted odds ratio=0.80), showed an inverse association between Ct seropositivity and fibroid prevalence in this population of African-American women. This is contrary to findings in previous studies [216,217]; however, these studies looked at older women. The authors suggest that bacterial infection can lead to regression of some tumour types [218] either by enhancing anti-tumour effects or by enhancing immune surveillance, particularly if infection coincided with fibroid progression. The authors suggest further research using animal models is required to elucidate the association between genital chlamydia infection and uterine fibrosis.

Frisse and collaborators used serology to ascertain the validity of self-reported Ct infection in a study of 409 English- or Spanish-speaking women between the ages of 18-35 years in the United States of America who were stopping contraceptives to attempt pregnancy [219] (Table 2.3). At baseline, a questionnaire was completed, a clinical examination was performed, and samples were collected for NAAT testing of NG, Ct, *Trichomonas vaginalis* (TV) and MG; blood samples were collected for serological testing for antibodies against Ct, TV and MG. As part of the medical questionnaire, participants were asked if they had ever been diagnosed by a health care professional as having had genital chlamydia. Blood samples were assayed using the modified MIF protocol [220]. Participants with either a serologically-confirmed or self-reported genital chlamydia were more likely to be younger, parous, non-white, unmarried, of lower socio-economic or educational level, uninsured, current drug user and former user of implant or Depo-Provera for contraception. There was only moderate agreement between self-reported Ct infection and seropositivity: 26% of participants reported a history of Ct infection, whereas 36% of participants were seropositive for antibodies against Ct. When comparing reported Ct infection to serological positivity, sensitivity was 52.1%, while specificity of self-reported infection to serology was 87.8%. Self-reporting was not found to be a valid marker of previous Ct infection, with only a moderate level of agreement between self-report and serology (Cohen's kappa=0.42, $p < 0.001$). The authors emphasized the need for assays with high validity for clinical assessment.

Summary of Seroprevalence Studies for Genital Chlamydia

From the surveys listed in the above section, it is clear that serological techniques have advanced and become more sensitive and specific. Due to the array of assays and antigens used, as well as

antibodies detected, it is not possible to compare results between most of the studies. MOMP and Pgp3 were the most common antigens, with IgG being the most common antibody detected. ELISAs, either commercially available or in-house, appear to be the assay of choice in the most recent studies, as they are robust, objective and are relatively high-throughput and can be automated. No multi-antigen assays were used in the studies described here, though they are currently being explored both with Ct-specific antigens and with a panel of antigens for STIs (T. Waterboer, personal communication). Numerous studies noted that participants were seropositive while not being aware of any prior Ct infections [60,90,219], and when participants of both genders were included, seroprevalence was higher in women than in men [88,200,212], which may be a result of greater sensitivity of the assay in women than in men [60,144,212] or that women experience a stronger immune reaction than men [95,212]. These could be further investigated by looking directly at the antibody levels present in males and females, rather than only seropositivity.

Table 2.3. Summary of genital Ct studies

Study	Location	Population (n)	Sample	Assay	Antigen	Immunoglobulin	Seropositivity
Darougar et al., 1980 [88]	London, UK	Healthy female (n=150) and male (n=150) blood donors	Serum	modified MIF	whole EBs: Ct serovars D-K	IgG- females	3.3% (1.4-7.6)
						IgG- males	0.67% (0.12-3.7)
						IgM- females	0.67% (0.12-3.7)
						IgM- males	0% (0-2.5)
Puolakkainen et al., 1987 [89]	Helsinki, Finland	Hospital patients of all ages with suspected viral infections (n=60,000)	Serum	CF	Lipopolysaccharide	complement	0.70% (0.64-0.77)
Jonsson et al., 1995 [90]	Ålidhem, Sweden	19-, 21-, 23- and 25-year old cohorts of women (n=529)	Cervical and urethral samples for culturing; serum	MIF	whole EBs: Ct serovars D-K	IgG	22.4% (19.2-26.0)
Satpathy et al., 2001 [92]	Delhi, India	Healthy male blood donors (n=844)	Serum	MIF	EBs- Ct serovars A-C	IgG	4.0% (2.9-5.6)
					EBs-Ct serovars D-K		8.5% (6.8-10.7)
					EBs- Ct serovars L1-L3		not specified
					EBs- Cp		42.8% (39.5-46.1)

Joyee et al., 2004 [200]	Tamil Nadu, India	Females (n=1,066) and males (n=783) aged 15-45 years, for a major study on community prevalence of sexually transmitted diseases (n=1849)	Urine samples for PCR, blood samples for serology	ELISA	Inactivated EB Ct antigen	IgM-females	3.3% (2.4-4.5)
						IgM-males	1.3% (0.7-2.3)
Baczynska et al., 2008 [93]	Denmark	Women requesting abortion (n=102, 100 women tested for antibodies)	Swabs to detect infection (PCR); serum	ELISA	MOMP	IgG	10.0% (5.5-17.4)
Horner et al., 2013 [102]	England	Unlinked residual samples from women, from Public Health England's Sero- Epidemiology Unit collection; samples from 1993-2010 (n=4,732)	Residual sera submitted to laboratories in England for routine microbiologi- cal or biochemical investigation s	in-house ELISA	Pgp3	IgG	17.5% (16.4- 18.6)
de Freitas et al., 2006 [94]	Cumaná, Estado Sucre, Venezuela	Pregnant women, 14- 43 years old attending	Serum			IgM	65.5% (54.8- 74.8)

		University Hospital "Antonio Patricio de Alcalá" (n=84)		Diagnostic Automation INC ELISA	"LGV type 2 broadly reacting antigen" [94]	IgA	19.1% (12.1-28.7)
van Aar et al., 2014 [95]	Netherlands	Men and women 15-39 years old participating in two population-based studies in 1996 and 2007; the 2007 study oversampled in migrants and low immunisation communities	Serum	Medac quantitative CT IgG ELISA	synthetic peptide from MOMP	IgG-men (1996)	5.2% (3.8-6.9)
						IgG-women (1996)	11.1% (9.2-13.6)
						IgG- men (2007)	6.2% (4.7-8.2)
						IgG- women (2007)	8.5% (6.8-10.6)
Ishak et al., 2015 [96]	Amazon region of Brazil	Blood samples of individuals from ten human population groups (n=1,710; 237 tested with MIF)	Blood	immunofluorescence assay (Biomerieux)	"serotype L2 of Ct"	IgG	50.2% (47.9-52.6)
				MIF	EB	IgG	8.7% (6.9-10.9)
					EB	IgM	2.6% (1.6-3.7)
Woodhall et al., 2016 [144]	England	Nationally-representative, population-based Health Survey for	Residual blood	in-house ELISAs- indirect and confirmatory,	Pgp3	IgG -women (2010/2012)	24.4% (22.0-27.1)

		England- men (n=1,119) and women (n=1,402) aged 16-44 2010-2012; samples from women aged 16-44 who participated in previous years (1994-2012) (n=3,361)		sandwich ELISA		IgG- men (2010/2012)	13.9% (11.8-16.2)
Horner et al., 2016 [60]	Dunedin, NZ	Male and female participants in the Dunedin Multidisciplinary Health and Development Study; samples collected at 26, 32 and 38 years of age (n=1,307)	Stored serum	in-house sandwich ELISA	Pgp3	IgG- females	24.1% (20.2-28.5)
						IgG- males	10.7% (8.1-13.9)
Righarts et al., 2017 [212]	Dunedin, NZ	Male (n= 448) and female (n=440) participants in the Dunedin Multidisciplinary Health and	Stored serum	Double-antigen sandwich ELISA	Pgp3	IgG- females (38 years)	26.8% (22.7-31.1)
						IgG- males (38 years)	13.1% (10.1-16.6)

		Development Study at age 38					
Moore et al., 2017 [213]	Detroit, USA	African-American females, aged 23-34 years, participating in the SELF study [221](n=1,587)	Serum	MIF	Whole EBs	IgG	57.2% (54.7-59.6)
Frise et al., 2017 [219]	USA	English- or Spanish-speaking women, aged 16-35, discontinuing contraceptive, with male partners and no known fertility problems (n=409)	Blood sample	Modified MIF [220]	Whole EBs	“anti- human globulin”	35.7% (31.2-40.5)

2.7 Discussion

Currently, several in-house and commercial assays exist for detecting antibodies against Ct. Although many commonly detect antibodies against Pgp3, assays are also available for detecting antibodies against MOMP and CT694. Assays also exist in a variety of forms: ELISAs, lateral flow assays and MBA to detect IgG, IgA and IgM. MIF is considered to have fallen out of favour and the summary above shows studies with conflicting results regarding cross-reactivity between Ct and Cp [90,92]. To-date, there has been little head-to-head comparison of the many assays, though this has been identified as a research priority [I. Toskin, personal communication]. There is a need for a head-to-head comparison of assays using a common set of serum standards to establish appropriate thresholds, sensitivity and specificity in a diverse array of patient groups, both for trachoma as well as genital chlamydia. The intended use of the assay will also dictate the required sensitivity and specificity. For example, assays with a higher threshold will likely be more specific and have a higher positive predictive value (PPV), which is desirable in post-intervention trachoma settings as over-estimating seropositivity may lead to continued, unnecessary, MDA efforts. However, a test used to screen for the sequelae of genital Ct infection may be more sensitive to effectively determine patients in need of further diagnostics.

The setting and use of the assay should be considered. For example, a Pgp3-based ELISA may be suitable for serosurveillance of anti-Ct antibodies to determine the cumulative incidence of genital Ct infections or as a proxy measure of the FoI in trachoma studies, whereas a multiplexed assay detecting antibodies against several immunogenic antigens would be useful in determining the risk of scarring sequelae, either of the tarsal conjunctiva or of the upper genital tract.

Some of these issues regarding assay selection are highlighted in Figure 2.8.

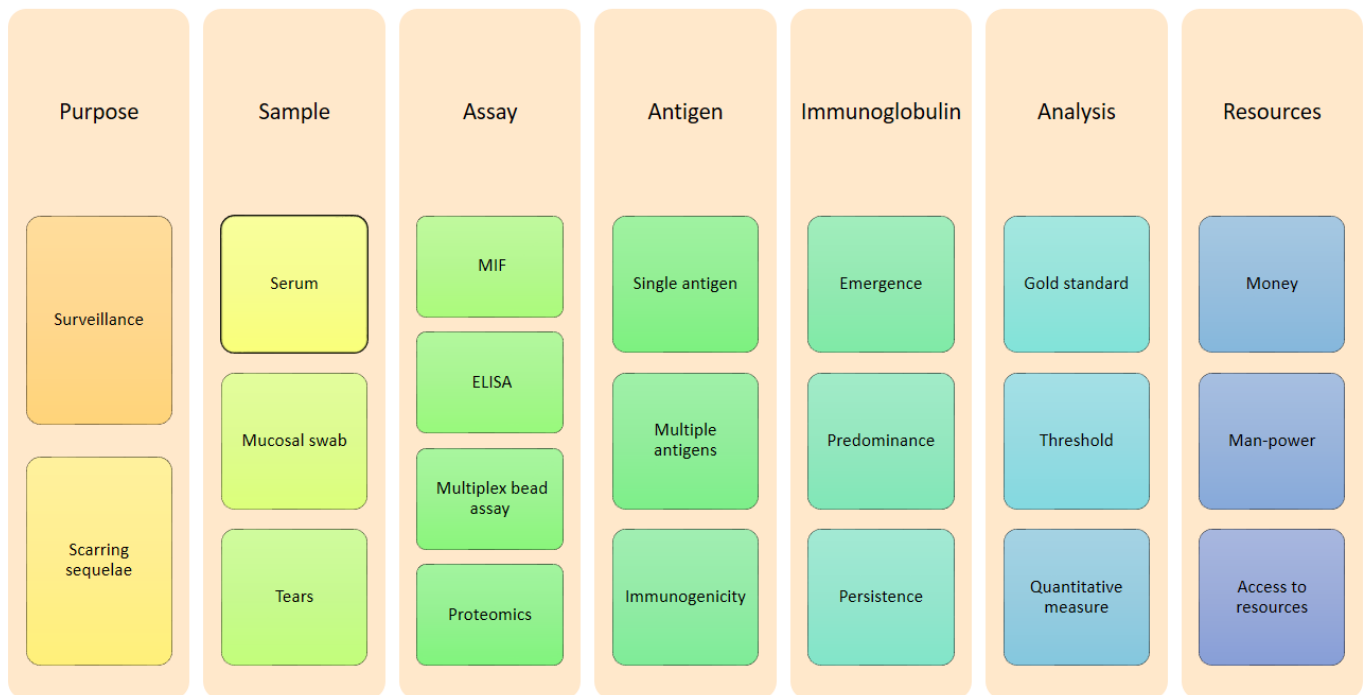


Figure 2.8. Factors to consider when selecting an assay to detect antibodies against *C. trachomatis*.

Much work remains to define the role of serology in public health, but it is clear that it has many exciting and potential applications. Further research should be focused on standardising assays using well-characterised serum samples. It is likely that different assays will be required for different purposes: an assay with a high sensitivity may be most appropriate as a screening tool, whilst one with a higher specificity may be more useful for measuring the sequelae of Ct infection or for estimating seroprevalence thresholds in trachoma elimination settings. The identification of immunogenic peptides specific for different disease conditions must be explored and a collection of well-characterised sera from a variety of sources is essential for future assay development and comparison between assays. Collaboration between academic research groups and public health bodies will be required to achieve this.

From a practical point of view, future work should focus on what is feasible in a programmatic context for population-based studies, although this may result in using less-robust methods, namely, a single-antigen ELISA rather than a multiplex assay and internally referenced thresholds rather than those based on ROC curves referring to infection testing. This may also result in estimates of seroprevalence based on binary positive/negative, but research should continue to explore the possibilities using the numerical output from assays and explore changes in antibody levels across ages and over time following initial Ct infection.

For identifying antigens indicative of scarring sequelae, the use of proteomics to identify additional antigens is necessary. Multiplexed assays are likely to be of more use than single antigen assays, and the additional cost more acceptable in this context. Appropriate reference samples will be challenging due to the multiple aetiologies associated with scarring of the upper genital tract in women, and the invasive nature of current diagnostic assays.

The production of a target product profile would help to guide further assay developments or refinements, while the development of a serum bank would facilitate comparison between assays and standardise assay evaluation. It is now up to international leaders in serology and chlamydia research to press for action.

2.8 References

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Chapter 3- Development of a Pgp3-specific ELISA to measure antibodies against Ct using fingerprick dried blood spots

Summary

In this chapter, the author outlines the development of a Pgp3-specific ELISA. The intent of this assay was to detect antibodies against a highly immunogenic protein expressed by the Ct plasmid. The assay was developed in collaboration with colleagues at the CDC, who developed a multiplex bead assay; the current assay requires highly specialised instrumentation which is often not available in trachoma-endemic populations. It was therefore the intent that this assay will be used 'on-site'. Indeed, this ELISA has been used in The Gambia, Ghana and Malawi, in addition to being used to test a large array of samples assayed at LSHTM.

This ELISA is based on the same Pgp3 from Ct serovar D-UW-3/.Cx used in the MBA [1] and in a whole-genome scale proteome assay [2]. It relies on five reference serum standards, with known levels of antibodies, measured in arbitrary units (u). The quantity of secondary antibody (HRP-conjugated mouse anti-human IgG) and the incubation time of TMB were optimised.

Significant quality control was undertaken. Reference standards were tested in triplicate with the standard deviation and coefficients of variation produced for each plate. As expected, the highest (1000u) and lowest (0 u and 50 u) standards showed more variation than those which fell in the linear range of the assay; a plate was permitted to have no more than one standard with a coefficient of variation greater than 15%. Standards were tracked across assays and inter-plate variation was less than 15%.

The ELISA developed here forms the basis for the serological analyses presented in Chapters 4, 5 and 6.

3.1 Purpose

To develop a Pgp3-specific indirect ELISA based on dried blood spots.

Previous work using serological assays to measure antibodies against Ct in trachoma-endemic populations was performed using a multiplex bead assay (MBA) and measured antibodies against Pgp3 and CT694 [1,3,4]. This assay was financially unviable for this thesis research thus with collaborators from the CDC, the protocol for a Pgp3-specific ELISA was optimised by the author [5]. Antigen was kindly provided by Prof Guangming Zhong and the author expressed and purified the protein while at the CDC in March 2013.

3.2 Background

A multiplex bead was developed by collaborators at the CDC. This assay measured antibodies against Pgp3 and CT694 [1]. Due to changes in funding sources, the required platform and reagents were deemed to be too expensive for this thesis research. Additionally, if serology is to be used programmatically as part of trachoma elimination programmes, protocols should be developed that can be used in country, rather than relying on testing by international institutes. Therefore, a Pgp3-specific assay was developed that was affordable [5] and could be run on an ELISA plate reader, which are available in many labs in trachoma-endemic countries [6].

3.3 Methods

The Pgp3-specific ELISA is an indirect ELISA. A detailed description of the finalised ELISA protocol is provided in Chapter 4. This chapter outlines the optimisation steps that were undertaken by the author prior to testing samples from the field.

In brief, Pgp3, a Ct-specific antigen, was coated to microtitre plates at known concentrations. Pgp3 was kindly provided by Professor Guangming Zhong of the Department of Microbiology and Immunology, University of Texas Health Science Center at San Antonio [2]. Protein was expressed and purified by the author while at the CDC in March 2013. Serum, in the form of eluted blood spots, was incubated so that anti-Pgp3 antibodies present in the serum bound to the antigen. Serum was then removed from the plate by washing with PBS + 0.3% v/v Tween-20 (PBSTw). An optimised concentration of horseradish peroxidase (HRP)-labelled mouse anti-human IgG(Fc)-HRP (Southern Biotech, Birmingham, USA) was applied and plates were incubated for one hour. Plates were washed to remove unbound antibodies. Fifty microliters of 3,3',5,5'-tetramethylbenzidine (TMB) (KPL, Gaithersburg, USA) were added and the mixture was incubated in the dark for an optimised amount of time at room temperature. The colorimetric reaction was stopped with 50 μ L 1N H₂SO₄ and

optical density was read at 450 nm (OD₄₅₀) on a Spectramax M3 plate reader (Molecular Devices, Wokingham UK). Figure 3.1 shows a schematic of the indirect ELISA set-up.

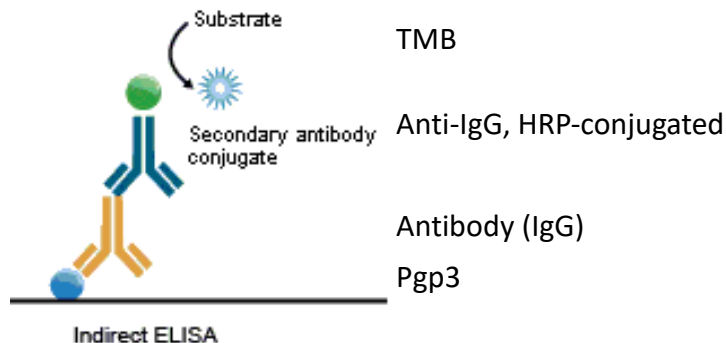


Figure 3.1. Visual representation of ELISA set-up (modified from www.abnova.com) Pgp3 is bound to the surface, where is capture anti-Pgp3 IgG present in the eluted dried blood spots bound to the antigen. The sample was then incubated with a secondary antibody which is conjugated with HRP and then TMB was added to produce a colourimetric reaction.

3.3.1 Standards

The standards were provided by the CDC and were prepared using serum from a Haitian donor, which tested at 2.0 OD₄₅₀. This serum has arbitrarily been assigned 1000 arbitrary units (u). The serum was diluted using a normal human serum from a blood bank donor; dilutions were made to 500 u, 200 u, and 50 u, while the normal serum itself was assigned 0 arbitrary units.

3.3.2 Optimisation of anti-IgG concentration

Anti-IgG bound to the IgG present in serum/samples, while the conjugated HRP (horseradish peroxidase) acted as an enzyme to oxidize TMB, causing a colourimetric change as the solution became blue.

The standard protocol was followed for the Pgp3 ELISA, with the concentration of anti-IgG-HRP changed. The standard concentration used was 1:1000 (5.5ul in 5.5ml PBSTw). The first column of the plate was incubated with the standard concentration, while each subsequent column was incubated with a decreasing serial dilution, down to 1:2048k (Figure 3.2). Standards 1000 u, 200 u, 0 u and Blank (PBSTw with milk) were tested in duplicate at each anti-IgG concentration, as shown below. TMB was incubated for 9 minutes before the colorimetric reaction was stopped with 50 µL 1N H₂SO₄d before being read on the plate reader.

	1:1k	1:2k	1:4k	1:8k	1:16k	1:32k	1:64k	1:128k	1:256k	1:512k	1:1024k	1:2048k
A	1000u											
B	1000u											
C	200u											
D	200u											
E	0u											
F	0u											
G	BL											
H	BL											

Figure 3.2. Microtitre plate layout for optimisation of HRP-conjugated anti-human IgG used in a Pgp3-specific ELISA. Known standards were tested in duplicate at each of 12 concentrations of anti-IgG.

Results are shown in Figure 3.3 and Table 3.1.

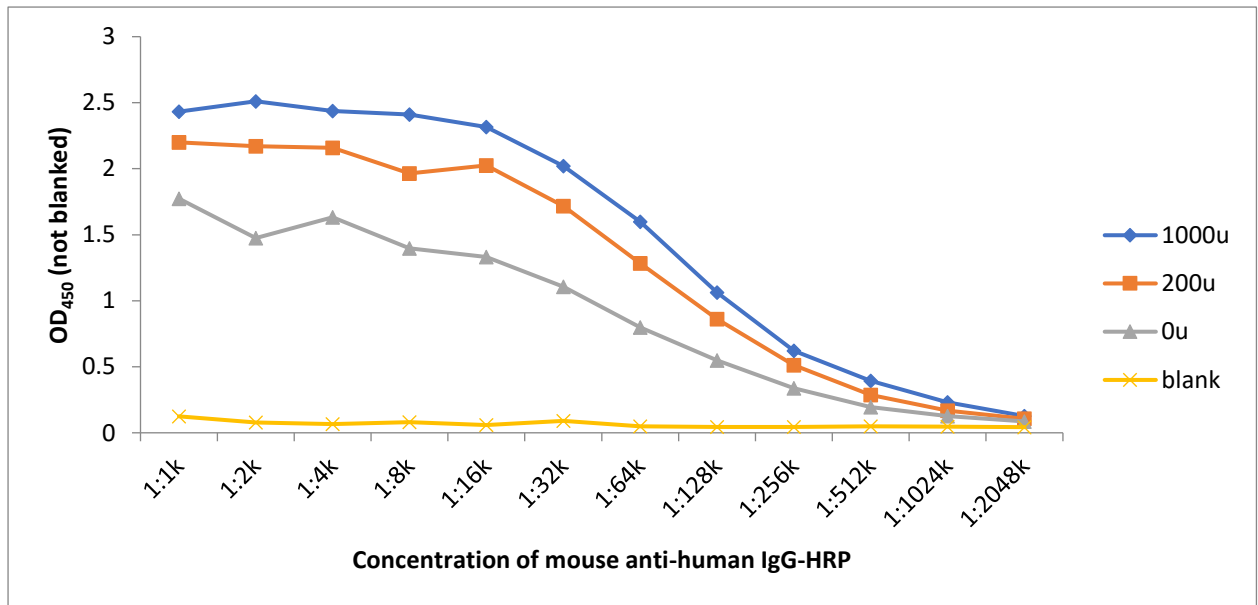


Figure 3.3. Serial dilution of HRP-conjugated mouse anti-human IgG. The standard concentration of anti-IgG is 1:1000 in PBS-Tween. Each well had 50µl of anti-IgG dilution applied, before incubating at room temperature for one hour.

Table 3.1 shows the ratios between standards. Looking at the ratios of the standards in the linear range (1:16k to 1:256k), the greatest difference between high and low is seen at 1:64k

Table 3.1. Ratio of OD values between standards measured to optimise the concentration of anti-IgG

	1000 u/0 u	1000 u/200 u	200 u/0 u
1:16k	1.74	1.14	1.52
1:32k	2.13	1.28	1.62
1:64k	2.00	1.24	1.61
1:128k	1.93	1.23	1.57
1:256k	1.84	1.21	1.52

3.3.3 Optimisation of TMB incubation time

As for the optimisation of anti-IgG concentration, the high-, mid-, and low- standards and blanks were tested in duplicate, at an anti-IgG concentration of 1:32k, while the incubation time increased from 4 to 15 minutes.

	4 min	5 min	6 min	7 min	8 min	9 min	10 min	11 min	12 min	13 min	14 min	15 min
A	1000u											
B	1000u											
C	200u											
D	200u											
E	0u											
F	0u											
G	BL											
H	BL											

Figure 3.4. Microtitre plate layout for optimisation of TMB incubation time used in a Pgp3-specific ELISA. Known standards were tested in duplicate at each of 12 lengths of time for incubation with TMB.

Results are shown in Figure 3.5 and Table 3.2

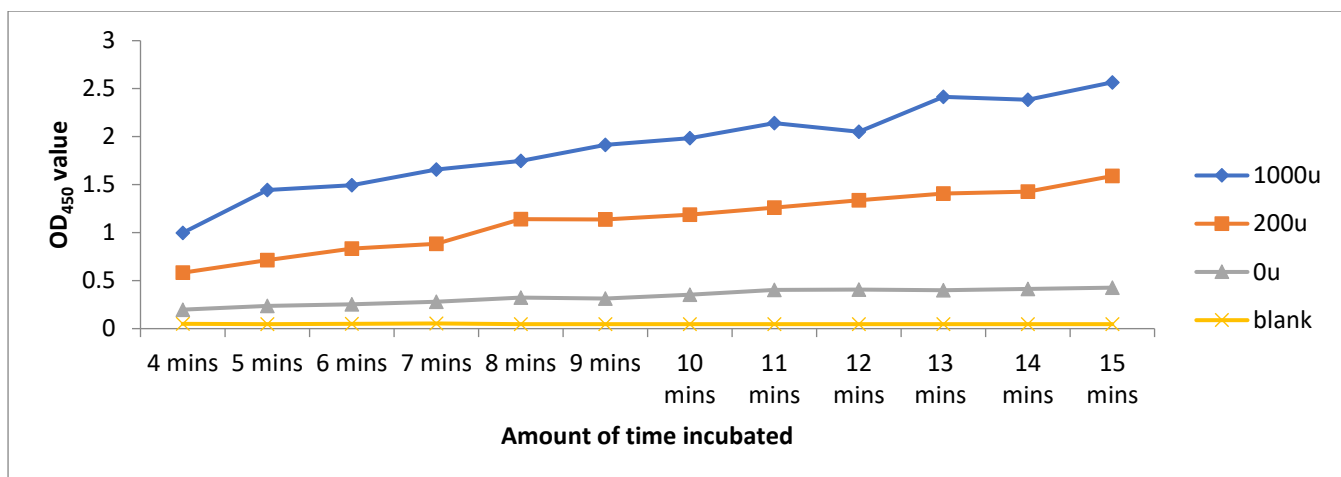


Figure 3.5. Optimisation of TMB incubation time for Pgp3-specific ELISA. Standard protocol was for four minutes incubation with TMB, however the best ratios between standards were at 9 minutes incubation.

Table 3.2 shows the ratios between standards. The highest ratios between standards occurred after 9 minutes of incubation.

Table 3.2. Ratio of OD₄₅₀ values between standards measured to optimise the incubation time with TMB.

Ratios	1000 u/200 u	200 u/0 u	1000 u/0 u
4 mins	2.98	2.94	5.04
5 mins	3.04	3.02	5.16
6 mins	3.27	3.28	5.88
7 mins	3.40	3.14	5.87
8 mins	3.46	3.53	5.40
9 mins	3.78	3.64	6.12
10 mins	3.64	3.35	5.59
11 mins	3.49	3.14	5.32
12 mins	3.17	3.27	5.03
13 mins	2.88	3.51	5.00
14 mins	2.07	3.45	4.77
15 mins	2.01	3.72	4.51

Based on the optimisation steps, the finalised protocol was to dilute anti-IgG to 1:32k and to incubate with TMB for 9 minutes.

3.3.4 Quality Control

The five serum controls (1000 u, 500 u, 200 u, 50 u, 0 u) were tested in triplicate and the mean values for each plate were tracked across each sample set and inter-plate variation was less than 15% across all plates in each sample set tested. A plate was permitted to have no more than one control with >15% variation from the sample set mean for that control; if a plate had two or more controls with values more than 15% greater or lesser than the sample set mean, the plate was re-run.

Less than 5% of plates were re-run due to variation. Additionally, the standards were run in triplicate and the coefficient of variation was calculated; in all cases this was less than 10% as shown in Figure 6. Coefficients of variation are less in the linear range of the standard curve; values that fall toward the top or bottom of the curve tend to have a higher amount of error because of both the assay's and plate reader's limits.

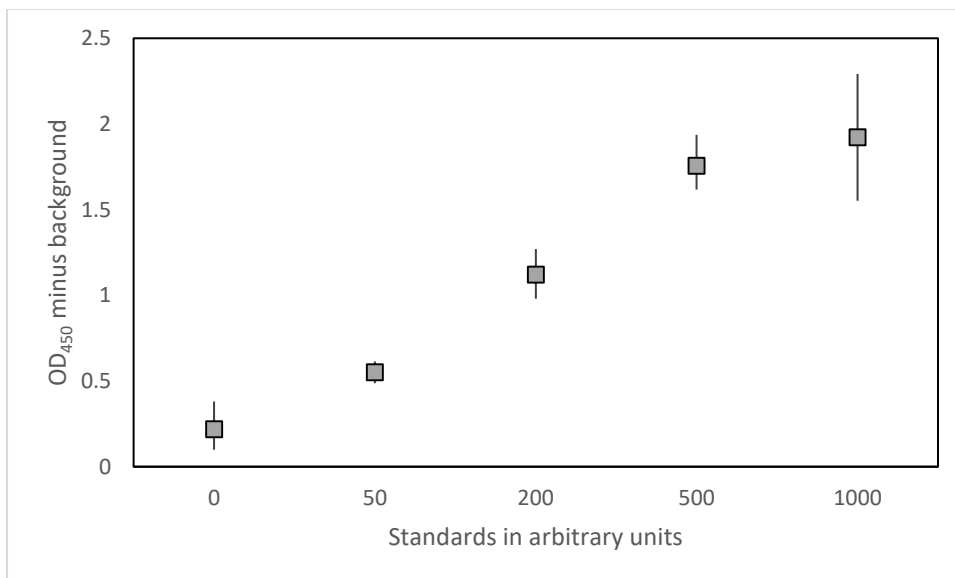


Figure 3.6. Standard curve produced from 5 standards at arbitrary units (1000 u, 500 u, 200 u, 50 u and 0 u). Error bars denote the coefficient of variation of the OD₄₅₀ values for each standard assayed in triplicate.

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Chapter 4 - *Defining seropositivity thresholds for use in trachoma elimination studies*



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SECTION A – Student Details

Student	Stephanie Migchelsen
Principal Supervisor	David Habyey
Thesis Title	Chlamydia trachomatis serology as a means of monitoring intervention activities to eliminate trachoma as a public health problem

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

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Where is the work intended to be published?	PLoS NTD
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Stage of publication	ready to be submitted

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I conducted the systematic review, produced the figures, wrote and edited the manuscript
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Student Signature: 
Supervisor Signature: 

Date: 10/09/18

Date: 12/9/18

Summary

In the previous chapter, the development of a Pgp3-specific ELISA was explained. In this chapter, the narrative focuses on appropriate means to set thresholds for positivity.

Both ELISAs and multiplex bead assays (MBA) produce continuous numerical readings as output for samples tested; ELISA results are measured in optical density (OD), while MBA results are median fluorescence intensity minus background (MFI-bkgd). It is appealing to assign a classification (seropositive or seronegative) to each sample assayed. This is challenging however, as the range of output value in the seropositive and seronegative populations will overlap to a lesser or greater extent.

In the following study, the author explored techniques of setting thresholds using data three sets of samples, each one from a population that was being assessed following trachoma prevalence surveys.

Previous studies have relied on Receive Operator Characteristic (ROC) curves; however, this runs the risk of reference standard misclassification if the references are inappropriately selected. To reduce the risk of setting an inappropriate seropositivity threshold, the author explored possible solutions based solely on data generated during the study. The author included a method based on visual examination of the data, to demonstrate that the threshold based on human pattern recognition is consistent with those set by more complex modelling techniques such as finite mixture modelling and expectation-maximisation algorithm. All three of these methods established similar thresholds.

In comparison, three thresholds were set using an ROC curve. Thresholds were set to maximise Youden's J-index to balance sensitivity and specificity, the second and third were set for high sensitivity (minimum 80%) and high specificity (minimum 98%), respectively. The thresholds set using the ROC curve were higher than those set using internal calibration and therefore were more specific but had reduced sensitivity.

Setting appropriate thresholds allows for the estimation of seroprevalence, a measure that can be easily understood. Furthermore, additional analyses, such as estimating changes in force of infection (Fol) over time depend on age-specific seroprevalence estimates. In a longitudinal context, however, the change in seroprevalence over time, from a survey prior to the commencement of MDA compared to a follow-up or monitoring survey may be of greater importance than the absolute seroprevalence.

The subsequent chapter relies on the seroprevalence estimates established in this chapter to estimate changes in the Fol in two districts in The Gambia.

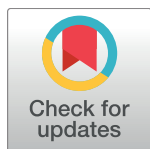
RESEARCH ARTICLE

Defining Seropositivity Thresholds for Use in Trachoma Elimination Studies

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Abstract

Background

Efforts are underway to eliminate trachoma as a public health problem by 2020. Programmatic guidelines are based on clinical signs that correlate poorly with *Chlamydia trachomatis* (Ct) infection in post-treatment and low-endemicity settings. Age-specific seroprevalence of anti Ct Pgp3 antibodies has been proposed as an alternative indicator of the need for intervention. To standardise the use of these tools, it is necessary to develop an analytical approach that performs reproducibly both within and between studies.

Methodology

Dried blood spots were collected in 2014 from children aged 1–9 years in Laos (n = 952) and Uganda (n = 2700) and from people aged 1–90 years in The Gambia (n = 1868). Anti-Pgp3 antibodies were detected by ELISA. A number of visual and statistical analytical approaches for defining serological status were compared.

Principal Findings

Seroprevalence was estimated at 11.3% (Laos), 13.4% (Uganda) and 29.3% (The Gambia) by visual inspection of the inflection point. The expectation-maximisation algorithm estimated seroprevalence at 10.4% (Laos), 24.3% (Uganda) and 29.3% (The Gambia). Finite mixture model estimates were 15.6% (Laos), 17.1% (Uganda) and 26.2% (The Gambia). Receiver operating characteristic (ROC) curve analysis using a threshold calibrated against

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Competing Interests: SG is employed by the commercial company IHRC, Inc. and is a contractor at the Centres for Disease Control and Prevention. The authors have declared that no competing interests exist.

external reference specimens estimated the seroprevalence at 6.7% (Laos), 6.8% (Uganda) and 20.9% (The Gambia) when the threshold was set to optimise Youden’s J index. The ROC curve analysis was found to estimate seroprevalence at lower levels than estimates based on thresholds established using internal reference data. Thresholds defined using internal reference threshold methods did not vary substantially between population samples.

Conclusions

Internally calibrated approaches to threshold specification are reproducible and consistent and thus have advantages over methods that require external calibrators. We propose that future serological analyses in trachoma use a finite mixture model or expectation-maximisation algorithm as a means of setting the threshold for ELISA data. This will facilitate standardisation and harmonisation between studies and eliminate the need to establish and maintain a global calibration standard.

Author Summary

Trachoma is caused by the bacterium *Chlamydia trachomatis* (Ct). Individuals who have previously been infected with Ct carry specific antibodies in their blood. Recent studies have suggested that these antibodies may be a good way to estimate the intensity of transmission of this bacterium in a population. Among people who do have antibodies (seropositives) there is variation in the amount that is detectable in their blood. Some people have such low levels that differentiating them from those who don’t have antibodies (seronegatives) is challenging. We used a new test for Ct antibodies on blood specimens from three countries. Our test worked extremely well, giving reproducible results when we tested the same samples multiple times. We compared four different methods for setting the position of the threshold line between seronegatives and seropositives. The estimated transmission intensity in each country varied depending on the threshold method used, but two methods that used statistical modelling algorithms to define the two groups performed consistently across all three countries’ samples. We recommend that future studies should consider adopting the statistical modelling approaches, as they are objective tests that require no reference material and allow for standardisation between studies.

Introduction

Trachoma is caused by ocular infection with the bacterium *Chlamydia trachomatis* (Ct) [1]. It is the leading infectious cause of blindness worldwide [2]. The World Health Organization (WHO) estimates that over 200 million people in 42 countries are at risk from trachoma blindness [3], that 1.4 million people experience moderate to severe visual impairment because of the disease and that of these, around 450,000 have been irreversibly blinded [4].

The most commonly used system for estimating the prevalence of trachoma uses the WHO simplified grading system [5] of clinical signs of trachoma. These include trachomatous inflammation—follicular (TF), trachomatous inflammation—intense (TI) and trachomatous trichiasis (TT), which is the rubbing of the eyelashes against the globe of the eye. WHO guidelines recommend the SAFE strategy to combat trachoma: Surgery to treat trichiasis, annual

mass-drug administration (MDA) of Antibiotics to treat Ct infection and Facial cleanliness and Environmental improvement to reduce transmission. Implementation of the SAFE strategy and cessation of MDA depends on the prevalence of TF in children aged 1–9 years. Concerns have been raised about the appropriateness of having treatment guidelines based on clinical signs such as TF and TI. In some low endemicity [6,7] and post-MDA settings [8,9], both TF and TI correlate poorly with the prevalence of Ct infection and both clinical signs are sometimes associated with bacteria other than Ct [10,11].

Tests for infection have been suggested as possible tools for trachoma control programmes. Numerous nucleic acid-amplification tests (NAATs) have been developed, including the adapted use of commercial kits originally designed for diagnosing genitourinary Ct infections [12–16]. NAATs have been shown to be cost-effective in some settings [17] but concerns have been raised that the per-sample cost of NAATs can be too much for national eye health programmes in countries where trachoma remains a problem [18]. The cost of specialist devices and platforms for deploying NAATs can also be prohibitive.

Serology has been suggested as a possible alternative to clinical signs and infection testing, as it indicates the cumulative exposure to Ct [19,20], with the potential to assess the impact of intervention efforts [21]. By monitoring the exposure to Ct of the youngest age groups, born after implementation of MDA, serology may prove useful for confirming that transmission has been interrupted [22].

Serology has recently been used in several studies [19,20,22–24], three of which have taken place in districts that have completed three or more rounds of MDA [22–24]. These studies have used the multiplex bead array platform (Bio-rad, Hercules, California) to detect antibodies against Pgp3 and CT694, antigens thought to be highly immunogenic [25]. Because this platform is costly, technically complex and unlikely to be found in most laboratories in resource-limited regions, alternative, simpler methods of antibody detection have been proposed [22,26].

To make serological testing more widely accessible, the Pgp3/CT694 assay used in previous studies [19,20,22–24] has been adapted for use in a simple Pgp3-specific enzyme-linked immunosorbent assay (ELISA). Pgp3 is a Ct-specific 84kDa heterotrimeric protein [27] and is recognised by specific IgG [28]. It is thought to be the most immunodominant of the proteins encoded by the Ct plasmid that is unique to Ct [29].

ELISAs are routinely used to detect specific IgG in dried blood spots [30–34]. ELISA data, measured as optical density (OD) is quantitative and continuous. It is desirable to be able to assign a classification (seronegative, seropositive) to each sample, but this can be challenging because the distributions of OD values in the negative and positive populations may overlap to a greater or lesser extent [34]. The aim of this study was to determine the most appropriate method for setting the threshold for positivity as well as to determine the usefulness of an anti-Pgp3-specific ELISA for identifying communities in which the transmission of ocular Ct has been interrupted. We tested dried blood spots collected as part of trachoma surveys in three countries: Laos, Uganda and The Gambia. We evaluated the age-specific seroprevalence using four methods and compared the resulting estimates of prevalence of seropositivity based on six possible thresholds. We discuss the merits of the different methods in the context of programmes seeking to monitor the elimination of trachoma as a public health problem.

Methods and Materials

Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. This study received approval from the Ethics Committee of the London School of Hygiene & Tropical Medicine

(LSHTM; references 6319, 6514, 8355, 8918), UK; the Ministry of Health of the Lao People's Democratic Republic (No:48 NIOPH/NECHR), Ugandan Ministry of Health (VCD-IRC/053) and The Gambia government/Medical Research Council (MRC) Joint Ethics Committee (SCC1408v2). In all countries, a local health official explained the study to each head of household, answered any questions and explained the written consent form before requesting their agreement and signature. Written (thumbprint or signature) consent was obtained from each participant or the parent or guardian of each child under 18 who participated; assent was sought from children aged 12–17.

Clinical assessment

Trachoma graders were trained according to the Global Trachoma Mapping Project (GTMP) protocols and were required to score a minimum kappa of 0.7 for the diagnosis of TF in an inter-grader agreement test with 50 eyes of 50 children [35,36]. The samples in Laos were collected in November 2014 as part of a follow-up study to the GTMP work completed there. Three districts in three regions were selected based on baseline trachoma survey findings that indicated potential 'hot spots' [37]. From these three regions, all children aged 1–9 in selected villages were invited to participate. Trachoma elimination programmes have never been undertaken in Laos. In Uganda, samples were collected as part of a trachoma impact survey in May 2014, following three years (2010–2012) of implementation of the A, F and E components of the SAFE strategy in two regions (Pader and Agogo). Prior to MDA, trachoma was considered highly endemic in these regions, although no data is publicly available. This study was a population based prevalence survey, which used a two stage sampling strategy; villages were selected with probability proportional to size, and households were randomly selected within each selected village based on a household list produced by the village chief and local health officials.

All children aged 1–9 years in the selected households were invited to participate. In The Gambia, a population based prevalence survey using a two stage sampling strategy was undertaken in February–March 2014; villages were selected with probability proportional to size, and households were randomly selected within each selected village based on a household list produced by the village chief and local health officials. One region, Lower River Region (LRR) had undergone three rounds of annual (2007–2009) MDA for trachoma, while the other, Upper River Region (URR), has never had trachoma elimination activities because trachoma has not been of a sufficiently high prevalence to justify implementation. All members of randomly selected households were invited to participate, regardless of age.

After informed consent was obtained, a trachoma grader examined both eyes for signs of trachoma using a binocular loupe (2.5×) and a torch. The grader changed gloves between each participant to minimise the risk of carry-over contamination. Antibiotics were provided to individuals with evidence of active trachoma and/or the affected household, according to each country's national policy.

Blood collection

Each participant had a finger-prick blood sample collected onto filter paper (Trop-Bio Pty, Townsville, Australia), using a sterile single-use lancet (BD Microtrainer, Dublin, Ireland). Each filter paper had six extensions, calibrated to absorb 10 µL of blood. Samples were air-dried for approximately five hours and then stored in individual Whirl-Pak plastic bags (Nasco, Modesto, California) with desiccant sachets (Whatman, Little Chalfont, UK) before being stored at -20°C.

All samples were shipped to LSHTM for testing.

ELISA analysis of anti-Ct-Pgp3 antibodies

Dried blood spots (DBS) were tested for antibodies against Pgp3. One whole filter paper extension per sample was eluted in 250 μ L PBS + 0.3% v/v Tween-20 (PBSTw) (Sigma-Aldrich, Dorset, UK) + 5% w/v non-fat milk powder (PBSTw-milk) (AppliChem, Maryland Heights, USA) overnight at 4°C. Immulon 2HB 96-well plates (VWR International, Lutterworth, UK) were coated with recombinant Pgp3 protein [19] overnight at 4°C (25ng per well in 0.1M sodium carbonate buffer, pH 9.6). Plates were washed with PBSTw to remove unbound protein, blocked with 100 μ L PBSTw for 1 hour at 4°C and washed two times. Control sera with known ratios of Pgp3 antibodies (1000 units, 500 units, 200 units, 50 units and negative control serum) and a blank consisting of PBSTw-milk were run on every plate. All samples and controls were tested in triplicate at a 1:50 dilution in PBSTw-milk. After 2 hours incubation on an orbital shaker at room temperature, wells were washed 5 times and 50 μ L of an HRP-labelled mouse anti-human IgG(Fc)-HRP (Southern Biotech, Birmingham, USA) diluted 1:32,000 was added. Plates were incubated for 1 hour on an orbital plate shaker at room temperature then washed 5 times to remove unbound antibody. Fifty microliters of TMB (KPL, Gaithersburg, USA) was added and the mixture was incubated in the dark for 9 minutes at room temperature. The reaction was stopped with 50 μ L 1N H₂SO₄ and optical density was read at 450 nm (OD₄₅₀) on a Spectramax M3 plate reader (Molecular Devices, Wokingham UK). Readings were corrected for background by subtracting the average absorbance of three blank wells containing no serum, using Softmax Pro5 software (Molecular Devices, Wokingham UK).

Data analysis

Blanked OD₄₅₀ values for samples and controls were normalised by dividing the mean of the three wells against the mean of 200 unit control included on each plate. This was done for each plate.

Data analysis for ELISA was performed separately and masked to the results of demographic and clinical information. Statistical analysis was carried out using R [38].

Defining seropositivity

We used four different methods for establishing a threshold for seropositivity: visual inspection of the inflection point (VIP), a finite mixture model (FMM) [39], the expectation-maximisation algorithm (EM) [40] and an receiver operating characteristic (ROC) curve based on previously-assayed dried blood spots from children in Tanzania [19]. There are as yet no accepted guidelines as to what level of sensitivity or specificity is required of a serological test; thus we referred to a previously published template [18] and established three possible thresholds from the ROC curve: one maximising specificity, one with a sensitivity greater than 80% [18] and one optimising the balance between sensitivity and specificity, by maximising Youden's J-index [41].

Visual inflection point (VIP)

We asked 12 arbitrarily selected non-laboratory staff and students at LSHTM to visually examine a simple plot of the sorted OD₄₅₀ data curves and determine the inflection point for each sample set. For this exercise, we defined the inflection point as the point on the data curve where the curve changes from predominantly horizontal to predominantly vertical. The 12 values were then averaged to determine the threshold and standard deviations (SDs) were calculated.

Finite mixture model (FMM)

A finite mixture model [42] was used to classify the samples as seropositive or seronegative based on normalised OD₄₅₀ values. The data were fitted using maximum likelihood methods, estimating the distribution parameters for each classification group (seropositive or seronegative) as well as the proportion of samples in each category to fit the overall distribution of results [34,43,44]. The threshold for seropositivity was then defined as the mean of the Gaussian distribution of the seronegative population plus three SDs of the seronegative population [44,45]. FMM was performed on each set of samples, based on country of origin.

Expectation-maximisation algorithm (EM)

The expectation-maximisation algorithm is similar to FMM in that it classifies samples based on population parameters. It relies on the Bayesian information criterion to select an appropriate model. EM is an iterative optimization method to estimate some unknown parameter [40], in this case the threshold between seropositive and seronegative, given the number of clusters and the normalised OD₄₅₀ values. EM estimates where to set the threshold while maximising the likelihood of each sample parameter [40]. Using the ‘mclust’ package in R, parameters were set to specify a univariate model with equal variance between 2 clusters [46].

Receiver operating characteristics (ROC) Curve

Serum samples from 122 children from the United States and blood spots from 11 Ct-specific PCR-positive children from Tanzania were used to make the original ROC curve [19]. A second set of 124 Tanzanian dried blood spots were assayed using the multiplex bead array and dichotomised based on the original threshold. These samples were then re-tested with the ELISA and the data from this assay were used to generate the ROC curve used in this manuscript. The R package ‘Epi’ [47] was used to generate three different thresholds: the first of which maximises Youden’s J-index to balance sensitivity and specificity [41], the second and third were set for high sensitivity (minimum 80%) and high specificity (minimum 98%), respectively.

Statistical analysis

The prevalence of signs of trachoma and the exact binomial confidence intervals were calculated using the R ‘Stats’ package [38]. Due to the low prevalence of clinical signs, Fisher’s exact test was used to test for association [48].

Seroprevalence in each population was calculated using each of six thresholds. We also examined the relationship between the clinical data and serological data. Due to the low prevalence of clinical signs, data for clinical signs were pooled across all three studies.

Results

Clinical assessment

We recruited 978 Laotian children aged 1–9 years from the provinces of Attapu (n = 406), Houaphan (n = 307) and Phôngsali (n = 239). Twenty-six participants had incomplete clinical records and were excluded from further study. The proportions of the sample populations who were male were 52.9%, 60.3% and 54.0% in Attapu, Houaphan and Phôngsali, respectively. The median age was five years in all three provinces. Fifteen cases of TF were diagnosed (1.6%, exact binomial CI = 0.9%–2.6%), 11 of which were bilateral cases (Table 1). No cases of TI were observed. There was a higher prevalence of TF in Attapu (2.7%, 11/406) than in either Houaphan (1.0%, 3/307) or Phôngsali (0.4%, 1/239), (p = 0.02) using Fisher’s exact test [49] with the Simes-Bonferroni correction for multiple tests [50].

2738 children aged 1–9 years were recruited in the Ugandan districts of Agogo (n = 1388, 49.7% male) and Pader (n = 1377, 50.4% male). 38 participants were missing complete clinical data and were excluded from further study. The median age was five years in both districts. 93 cases of TF were diagnosed (3.4%, exact binomial CI = 2.8%-4.2%), 44 of which were bilateral. Eight cases of TI were diagnosed (0.3%, exact binomial CI = 0.1%-0.6%) (Table 1). No other clinical signs were assessed. The prevalence of TF was 3.2% in Agogo and 3.7% in Pader. There was no significant difference between the estimated prevalence of TF in the two districts (TF: $X^2 = 0.429$, $p = 0.5125$; TI: $X^2 = 3.1566$, $p = 0.07562$).

In the Gambia we recruited participants of all ages from the Lower River Region (LRR, n = 1028, 41.9% male) and Upper River Region (URR, n = 840, 42.5% male). Ten participants were excluded from the study because they either declined to provide a blood sample (n = 1) or had incomplete clinical data (n = 9). The median age in LRR was 13 years (range: 1–88) and 11 years in URR (range: 1–90). Overall, 30 cases of TF were diagnosed (1.6%, exact binomial CI = 1.1%-2.3%), 19 of which were bilateral (Table 1). There were 25 cases of TF in children aged 1–9 years. Four cases of TI were observed (0.2%, exact binomial CI = 0.06%-0.6%), two of which were in children aged 1–9 years. Examiners found 78 cases of TS (4.2%, exact binomial CI = 3.3%-5.2%), eight cases of TT (0.4%, exact binomial CI = 0.2%-0.8%) and one case of CO (0.05%, exact binomial CI = 0.001%-0.3%). There was a significant difference in TS prevalence between the URR and LRR ($X^2 = 7.2435$, $p = 0.007116$); the difference in TF prevalence was non-significant ($X^2 = 0.1343$, $p = 0.714$). The prevalence of TI, TT and CO in this population was too low for meaningful statistical analysis.

Observed frequencies of clinical signs of trachoma in the various samples are summarised in Table 1. A more detailed description, including prevalence by age and gender, is presented in Supplementary S1, S2 and S3 Tables.

Serological analysis

The five serum controls were tested in triplicate and the mean values for each plate were tracked across each sample set. The coefficient of variation was less than 10% in each of the

Table 1. Distribution of participants in three trachoma studies, including clinical signs.

Country	Province	N	TF	TI	TS	TT	CO
Laos*		952	15 (1.6%)	-	-	-	-
	Attapu	406 (42.6%)	11 (2.7%)	-	-	-	-
	Houaphan	307 (32.2%)	3 (1.0%)	-	-	-	-
	Phôngsali	239 (25.1%)	1 (0.4%)	-	-	-	-
Uganda*		2700	93 (3.4%)	8 (0.3%)	-	-	-
	Agogo	1353 (50.1%)	43 (3.2%)	1 (0.1%)	-	-	-
	Pader	1347 (49.9%)	50 (3.7%)	7 (0.5%)	-	-	-
The Gambia*		1868	30 (1.6%)	4 (0.2%)	78 (4.2%)	8 (0.4%)	1 (0.1%)
All	LRR	1028 (55.0%)	18 (1.8%)	4 (0.4%)	55 (5.4%)	7 (0.7%)	1 (0.1%)
	URR	840 (45.0%)	12 (1.4%)	0 (0.0%)	23 (2.7%)	1 (0.1%)	0 (0.0%)
1–9 year olds	LRR	383 (20.5%)	14 (3.7%)	2 (0.5%)	1 (0.3%)	0 (0.0%)	0 (0.0%)
	URR	359 (19.2%)	11 (3.1%)	0 (0.0%)	6 (1.7%)	0 (0.0%)	0 (0.0%)
≥10 year olds	LRR	645 (34.5%)	4 (0.6%)	2 (0.3%)	54 (8.4%)	7 (1.1%)	1 (0.2%)
	URR	481 (25.7%)	1 (0.2%)	0 (0.0%)	17 (2.6%)	1 (0.2%)	0 (0.0%)

*Age range in Laotian and Ugandan participants was 1–9 years; age range in all Gambian participants was 1–90 years; '-' not assessed.

N = Normal; F = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal opacity. LRR = Lower River Region; URR = Upper River Region.

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Table 2. The mean OD₄₅₀ value for the five controls sera used on the ELISA plates. Mean, SD and coefficient of variation for the five serum standards run alongside the Ugandan samples across 24 plates. Data were similar for the standards run alongside the Laotian and Gambian samples.

Control serum	Mean	SD	Coefficient of variation	Upper limit (mean+15%)	Lower limit (mean-15%)
1000u	2.01 OD ₄₅₀	0.13 OD ₄₅₀	6.47%	2.26 OD ₄₅₀	1.75 OD ₄₅₀
500u	1.74 OD ₄₅₀	0.13 OD ₄₅₀	7.38%	2.00 OD ₄₅₀	1.49 OD ₄₅₀
200u	1.11 OD ₄₅₀	0.10 OD ₄₅₀	9.46%	1.31 OD ₄₅₀	0.90 OD ₄₅₀
50u	0.63 OD ₄₅₀	0.06 OD ₄₅₀	9.45%	0.74 OD ₄₅₀	0.51 OD ₄₅₀
Negative control serum	0.28 OD ₄₅₀	0.02 OD ₄₅₀	8.51%	0.32 OD ₄₅₀	0.23 OD ₄₅₀

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three replicates of each control specimen. Inter-plate variation of controls was less than 15% across all plates in each sample set as shown in Table 2. A plate was permitted to have no more than one control with >15% variation from the sample set mean for that control; if a plate had two or more controls with values more than 15% greater or lesser than the sample set mean, the plate was re-run. Less than 5% of plates were re-run due to this. Table 2 shows the mean values and the accepted 15% range for the five controls.

The sample set for each country was tested separately. Each plate showed a large but narrowly distributed proportion of low-OD specimens, with a smaller proportion of higher-OD specimens. Fig 1 shows typical results from an ELISA plate. In all three sample sets, density data peak around 0.25 OD₄₅₀; this can be seen in centre panels B in Figs 2, 3 and 4.

Visual inflection point (VIP)

The leftmost panels of Figs 2A, 3A and 4A were shown to 12 people, each of whom was asked to determine each graph's point of inflection. The mean of the inflection points was calculated for each sample set and the SD and range were calculated. For Laos, the mean threshold was calculated to be 0.619 OD₄₅₀ (SD = 8.2%, range 0.485–0.750); for Uganda the threshold was calculated to be 0.641 OD₄₅₀ (SD = 14.4%, range 0.410–0.795) and for The Gambia the threshold was calculated to be 0.579 OD₄₅₀ (SD = 7.3%, range 0.402–0.673). The sorted normalised OD₄₅₀ values are shown in Figs 2A, 3A and 4A (leftmost panels), alongside marginal density distribution plots of the same values (centre panels) and boxplots (rightmost panels) showing the range of the 12 threshold values that were selected by the volunteers.

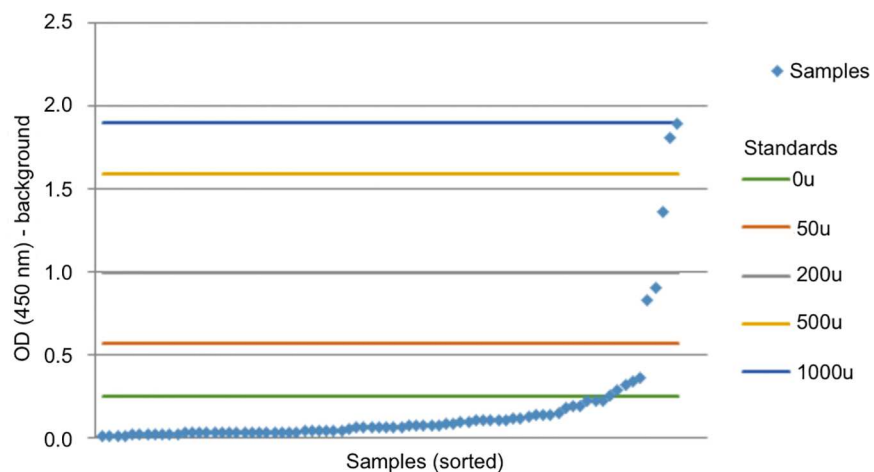


Fig 1. Typical results from an ELISA plate. Specimens are sorted by increasing OD values and are each represented by a separate diamond. The mean values of the controls tested in triplicate are represented by coloured horizontal lines.

doi:10.1371/journal.pntd.0005230.g001

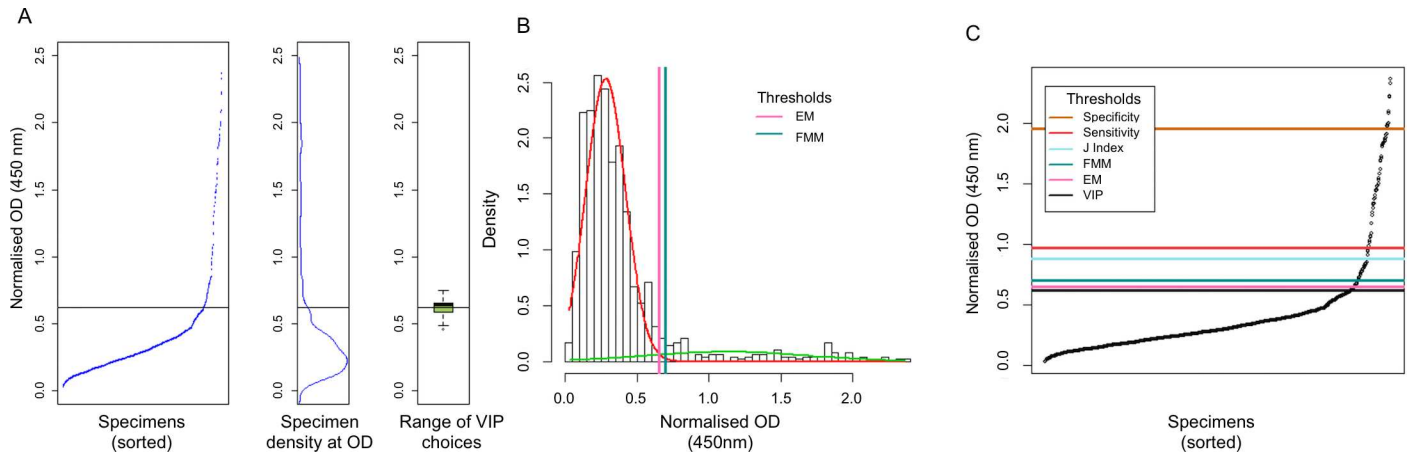


Fig 2. Threshold values for Laos (1–9 year olds) data. Panel A shows the threshold as determined by visual inflection point analysis by 12 volunteer individuals. Volunteers had access only to the data presented in the leftmost panels, which shows sorted OD₄₅₀ values. The second panel in A shows the density of data points for the sample while the third panel in A shows a box and whisker plots with the range of threshold values that were selected by the 12 volunteers. The box shows the inter-quartile range for the values, with the thick horizontal line marking the median value. Whiskers show the upper quartile plus 1.5x the range between the 1st and 3rd quartiles. Outliers are shown by an open circle. Panel B shows the thresholds set by the finite mixture model and expectation-maximisation algorithm. Density plots of normalised OD values and thresholds, showing the FMM estimated distribution functions of ‘seronegative’ specimens in red and ‘seropositive’ specimens in green. Vertical lines show the threshold values determined by the finite mixture model (right-most line) and the expectation-maximisation algorithm (left-most lines). Panel C compares the threshold specifications by four different methods. Scatterplots show the normalised and sorted OD₄₅₀ values with horizontal lines marking the thresholds specified by VIP (OD₄₅₀ = 0.619), EM (OD₄₅₀ = 0.650), FMM (OD₄₅₀ = 0.696), ROC curve maximising Youden’s J-index (OD₄₅₀ = 0.870), ROC curve with sensitivity >80% (OD₄₅₀ = 0.968) and ROC curve with specificity >98% (OD₄₅₀ = 1.951).

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Finite mixture model (FMM)

A finite mixture model was tested on all three sample sets, setting the threshold at the mean of the seronegative population plus three SDs [44,45]. The thresholds were set at 0.6963 OD₄₅₀, 0.5537 OD₄₅₀ and 0.6725 OD₄₅₀ for Laos, Uganda and The Gambia, respectively. The FMMs are shown in Figs 2B, 3B and 4B.

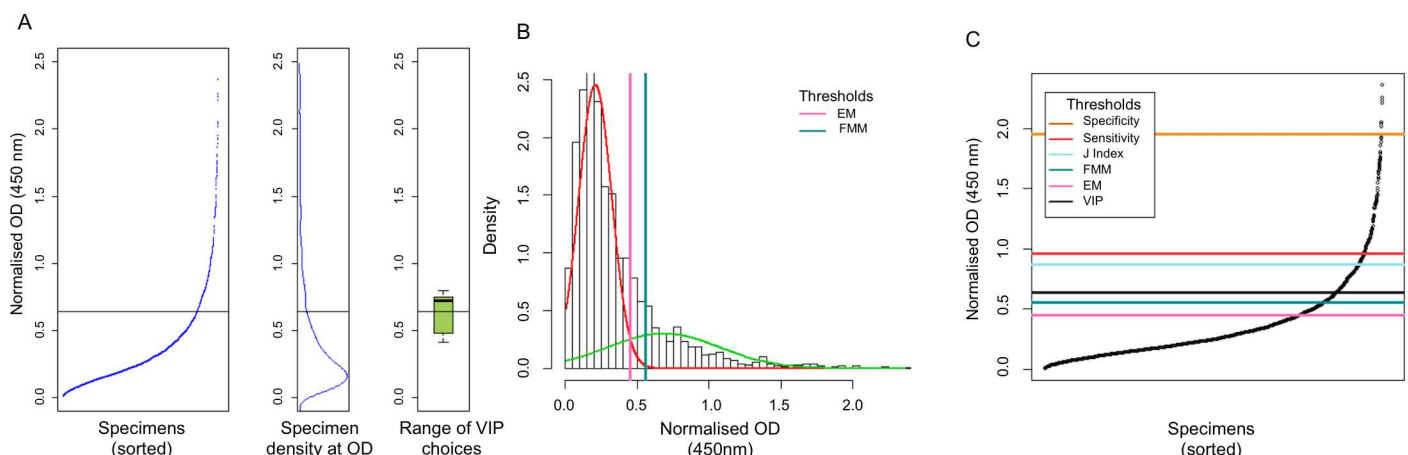


Fig 3. Threshold values for Uganda (1–9 year olds) data. Panel A shows the threshold as determined by visual inflection point analysis by 12 volunteer individuals, as detailed in Fig 2. Panel B shows the thresholds set by the finite mixture model and expectation-maximisation algorithm, as described in Fig 2. Panel C compares the threshold specifications by four different methods. Scatterplots show the normalised and sorted OD₄₅₀ values with horizontal lines marking the thresholds specified by VIP (OD₄₅₀ = 0.641), EM (OD₄₅₀ = 0.450), FMM (OD₄₅₀ = 0.554), ROC curve maximising Youden’s J-index (OD₄₅₀ = 0.870), ROC curve with sensitivity >80% (OD₄₅₀ = 0.968) and ROC curve with specificity >98% (OD₄₅₀ = 1.951).

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Expectation-maximisation algorithm (EM)

An EM model was fitted to all three sample sets, specifying parameters for a univariate model with equal variance between 2 clusters [45]. The thresholds were set at 0.65 OD₄₅₀, 0.45 OD₄₅₀ and 0.57 OD₄₅₀ for Laos, Uganda and The Gambia, respectively. The EM-derived threshold selections are shown in Figs 2B, 3B and 4B.

ROC curve

Using the ROC curve to set a threshold optimising Youden's J-index to balance specificity and sensitivity resulted in a threshold at 0.870 OD₄₅₀ (specificity 93.9%, sensitivity 91.4%). Setting the threshold to ensure a minimum sensitivity of 80% resulted in a threshold at 0.965 OD₄₅₀ (specificity 94.8%, sensitivity 89.4%). Setting the threshold for a minimum specificity of 98% resulted in a threshold at 1.951 OD₄₅₀ (specificity 98.28%, sensitivity 43.94%). Fig 5 shows the ROC curve with the three thresholds identified.

Panels 2C, 3C and 4C show all six thresholds in relation to the normalised OD₄₅₀ data in each of the three populations. The internally calibrated methods (i.e., VIP, FMM and EM) were reasonably conformant and appeared to favour threshold placements that were substantially lower than those set by the ROC, which is calibrated with Tanzanian specimens, even when a higher sensitivity (i.e., lower threshold value) test was specified in the ROC analysis. As a consequence of this, the seroprevalence estimates that were determined by VIP, EM and FMM were similar to one another, while the seroprevalence estimates set by any of the ROC curve thresholds were much lower in all three populations (Table 3).

Seroprevalence for each sample set, using the six different thresholds were calculated, along with 95% confidence intervals. As the threshold increases in value, fewer specimens are classified as being seropositive, decreasing the seroprevalence. The seroprevalence for each sample set at each threshold is presented in Table 3. Seroprevalence for each country by sex, region and age is provided in Supplementary S4, S5 and S6 Tables.

Table 4 presents the proportion of seropositive samples by clinical grade, as estimated by each threshold specification. Due to the relatively low prevalence of all clinical signs, prevalence values for have been pooled.

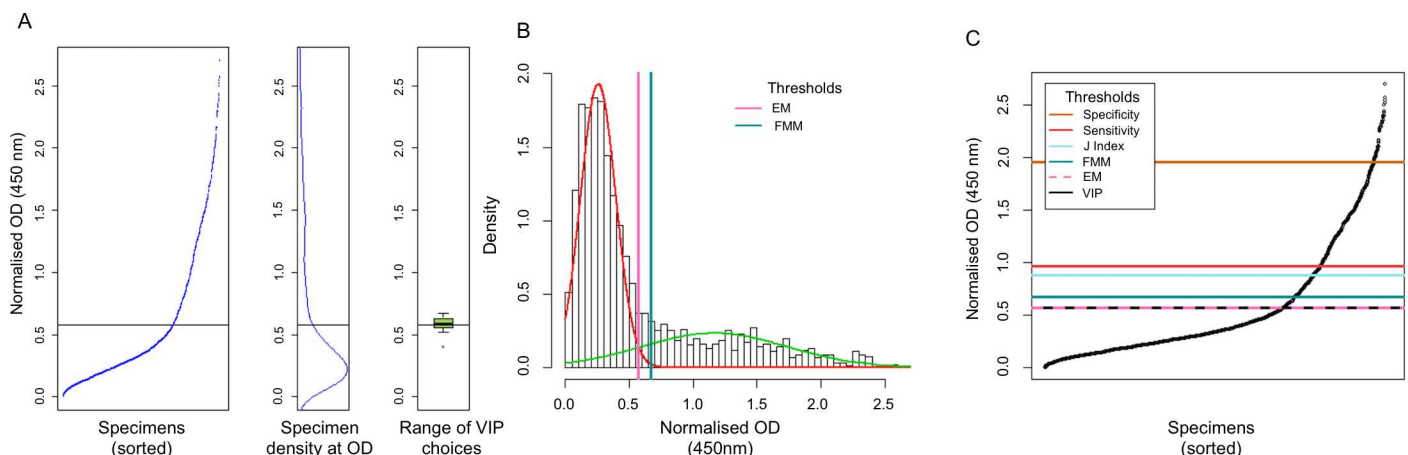


Fig 4. Threshold values for Gambian (all ages) data. Panel A shows the threshold as determined by visual inflection point analysis by 12 volunteer individuals, as detailed above in Fig 2. Panel B shows the thresholds set by the finite mixture model and expectation-maximisation algorithm, as described in Fig 2. Panel C compares the threshold specifications by four different methods. Scatterplots show the normalised and sorted OD₄₅₀ values with horizontal lines marking the thresholds specified by VIP (OD₄₅₀ = 0.570), EM (OD₄₅₀ = 0.570), FMM (OD₄₅₀ = 0.672), ROC curve maximising Youden's J-index (OD₄₅₀ = 0.870), ROC curve with sensitivity >80% (OD₄₅₀ = 0.968) and ROC curve with specificity >98% (OD₄₅₀ = 1.951). Note that the thresholds set by VIP and EM are identical (0.570 OD₄₅₀) and overlap on the graph.

doi:10.1371/journal.pntd.0005230.g004

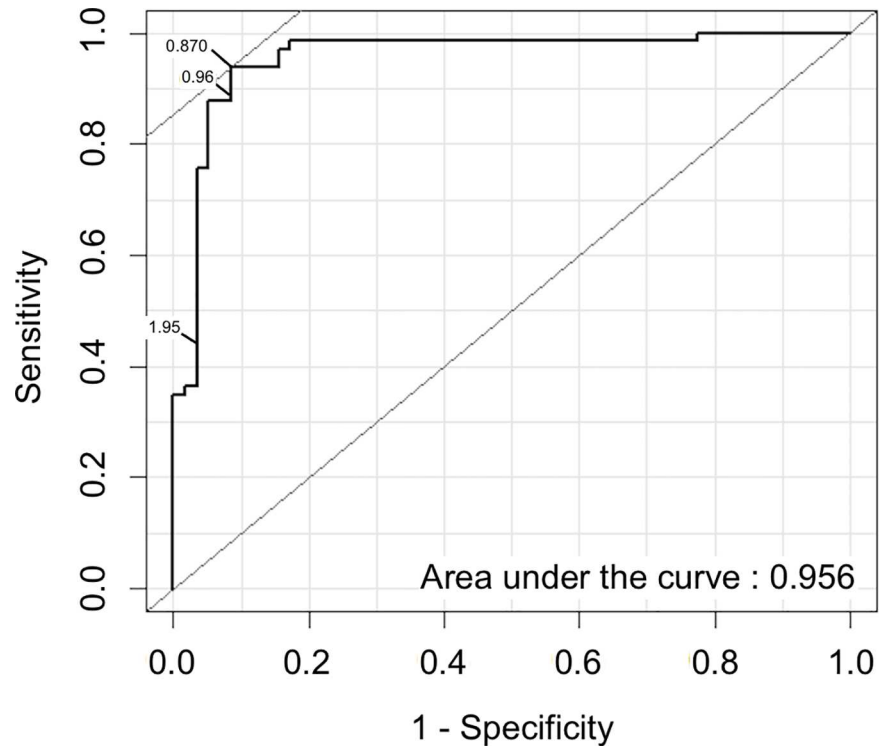


Fig 5. Receiver Operating Characteristic (ROC) curve showing the relationship between sensitivity, specificity and threshold values. Three different thresholds were specified to meet the requirements of: (A) an assay (threshold = 0.870 OD₄₅₀, specificity = 93.9%, sensitivity = 91.4%, PPV = 89.8%, NPV = 92.4%) with balanced sensitivity and specificity (maximal Youden's J value); (B) an assay (threshold = 0.965 OD₄₅₀, specificity 94.8%, sensitivity = 89.4%) with at least 80% sensitivity and (C) an assay (threshold = 1.951 OD₄₅₀, specificity = 98.3%, sensitivity = 43.9%, PPV = 66.7%, NPV = 95.0%) with at least 98% specificity.

doi:10.1371/journal.pntd.0005230.g005

Discussion

Several previous studies have used anti-Pgp3-specific ELISAs to test for genital chlamydial infection [21,51–54] but only one [55] has used the method for the detection of antibodies against ocular chlamydial infection. In this study, we used an ELISA test to detect IgG antibodies specific to the Ct protein Pgp3 in studies with large sample sizes from three countries. To

Table 3. Seroprevalence by Country, as estimated using alternate threshold specification methods.

	N	Threshold % (95% Confidence Interval)											
		VIP		EM		FMM		ROC Youden's J-index		ROC Sensitivity >80%		ROC Specificity > 98%	
		OD = 0.619		OD = 0.65		OD = 0.696		OD = 0.870		OD = 0.965		OD = 1.951	
Laos (1–9 year olds)	952	11.3%	(9.4–13.6)	10.4%	(8.6–12.6)	15.6%	(13.3–18.0)	6.7%	(5.3–8.6)	6.3%	(4.9–8.1)	1.1%	(0.5–2.0)
		OD = 0.641		OD = 0.45		OD = 0.5537							
Uganda (1–9 year olds)	2700	13.4%	(12.1–14.7)	24.3%	(22.7–26.0)	17.1%	(16.0–18.9)	6.8%	(5.9–7.8)	5.3%	(4.5–6.2)	0.3%	(0.1–0.8)
		OD = 0.57		OD = 0.57		OD = 0.672							
The Gambia (all ages)	1868	29.3%	(27.3–31.5)	29.3%	(27.3–31.5)	26.2%	(24.2–28.2)	20.9%	(19.1–22.9)	18.9%	(17.2–20.8)	3.3%	(2.6–4.3)

Seroprevalence by Gender, Region and Age is provided in Supplementary S2 Table.

VIP = visual inflection point; EM = expectation-maximisation algorithm; FMM = finite mixture model; ROC = receiver-operating characteristic curve.

OD = optical density, measured at 450 nm.

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date, this is the largest study to measure antibodies to Ct in trachoma-endemic populations and the first to look at populations from more than one country, including East Africa, West Africa and Southeast Asia. We have shown that within and between runs there is a low coefficient of variation in the assay and that the bimodal data distribution of normalised OD₄₅₀ values in those samples reflects that which would be expected in populations where a minority of individuals are seropositive and where there is a broad range of antibody titres in the seropositive sub-population. This is best observed in the data from the Gambia (Fig 4), where we included adults in the sample and where the more substantial seropositive sub-population can be accounted for by both sexually transmitted Ct infection and the formerly high level of endemicity of trachoma in the Gambia.

Clinical specimens without any Ct-specific IgG still have some degree of baseline reactivity in ELISA tests because of non-specific binding of irrelevant antibodies. There is also substantial between-specimen variation in seropositives, which reflects natural variation in the antibody titre. The potential for there being substantial overlap between the seronegative specimens with high baselines and the seropositives with low anti-Pgp3 antibody titres means that it can be difficult to differentiate between the two groups.

There is very little published information on the prevalence of trachoma in Laos and Uganda [56], but on the evidence of our analysis, clinical signs of disease are rare and the levels of seropositivity appear to be comparable to those in The Gambia, where elimination has been declared. We have no data on the prevalence of Ct infection in the communities in Laos and Uganda, nor is there any longitudinal data to monitor changes in antibody levels following documented infection. Numerous studies have looked at the prevalence of ocular Ct infection in The Gambia and shown it to be negligible [7,57,58]. All the populations we studied have received MDA and we did not screen a population with higher prevalence levels. Further research in meso- and hyper-endemic populations will be needed in order to assess the utility of this method in other settings.

We have shown how the method that is selected for the statistical interpretation of ELISA data (with particular regard to the method of threshold specification) can greatly change the population prevalence estimates that are derived. Methods that indicate the use of a higher threshold value are likely to be more specific and have a higher positive predictive value

Table 4. Proportion of participants with different phenotypes considered seropositive by each threshold.

	No sign of trachoma according to the WHO simplified system				Active trachoma		Scarring trachoma	
	% (95% confidence interval)				(TF and/or TI)		(TS and/or TT and/or CO)	
	1–9 year-olds		10+ year-olds		% (95%CI)		% (95%CI)	
N across all studies	4268		964		150		87	
VIP*	11.1	(10.2–12.1)	42.8	(39.7–46.0)	9.3	(5.4–15.5)	70.1	(59.2–79.2)
EM*	17.1	(16.0–18.2)	42.8	(39.7–46.0)	12	(7.5–18.6)	70.1	(59.2–79.2)
FMM*	12.7	(11.8–13.8)	38.5	(35.4–41.6)	16.7	(11.3–23.8)	69	(58.0–78.2)
J-index	5.8	(5.1–6.5)	31.8	(28.9–34.9)	4	(1.6–8.9)	55.2	(44.2–65.7)
Sensitivity >80%	4.7	(4.1–5.4)	28.8	(26.0–31.8)	3.3	(1.2–8.0)	49.4	(38.6–60.2)
Specificity >98%	0.4	(0.2–0.7)	4.8	(3.6–6.4)	0	(0–3.1)	16.1	(9.4–25.9)

*Note that country-specific thresholds were used for VIP, EM and FMM.

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal opacity.

VIP = visual inflection point; EM = expectation-maximisation algorithm; FMM = finite mixture model.

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(PPV), but they do incur a penalty in the form of reduced sensitivity. In the context of post-MDA trachoma control, a test with high PPV is more desirable as over-diagnosis might lead to the inappropriate continuation of MDA interventions. Meanwhile a lower sensitivity test, applied in a low prevalence setting such as the post-MDA population of the Gambia, is likely to have a high negative predictive value (NPV) and the clinical impact of the false negative rate is likely to be modest as long as the sensitivity does not fall too far. In our hands, the ROC analysis supported the use of higher thresholds than did the other methods. Unfortunately the reference material was not sampled from any natural population and so the estimated sensitivity and specificity of the test based on ROC were unlikely to reflect the true performance in the populations that were sampled in this study [59].

We explored three internally calibrated thresholding methods (i.e. using only data generated during the study), all of which specified thresholds at approximately the same OD_{450} value. This was true across sample sets from all three countries. It is perhaps unsurprising that similar estimates emerged from FMM and EM, as there are methodological similarities in the two approaches. At face value the VIP method might seem arbitrary and crude, but the human brain can outperform computers in some aspects of pattern recognition and by obtaining a threshold estimate that closely matches that of EM and FMM, our data indicate that the results of a conditionally independent method (VIP) correlate closely with the computational approaches and are able to successfully determine where the most obvious bimodal split in the data occurs. What gives FMM and EM the edge over VIP is that they are more replicable and that the different requirements for higher or lower specificity and sensitivity in different clinical settings can be controlled by changing the number of SDs that the algorithm uses to determine the cut point. For instance, an increasingly specific test could be implemented by setting the threshold at four, five or six SDs of the negative population, rather than three SDs we used here. None of the populations that we surveyed would be expected (based on clinical signs) to have a high level of Ct seropositivity and it may be that the data in Tables 3 and 4 (and Supplementary Data S4, S5 and S6 Tables) reflect a high false positive rate, low positive predictive value. By adjusting the parameters of the algorithms we might achieve a prevalence estimate that is more accurate, but without any gold standard we can never truly assess how accurate our estimates are. In the Gambian data, using respectively 4 or 5 SDs would have led to cut points at respectively $OD = 0.81$ and $OD = 0.95$, values much closer to the cut-points recommended by the ROC analysis.

For programmatic purposes, the absolute value and accuracy of the prevalence estimate is actually somewhat less important than the precision of that estimate and the longitudinal change in repeat measures from the same population across the lifetime of the intervention and monitoring programme. This is because the absolute estimate is clearly highly variable given quite arbitrary choices made during data analysis, whilst percentage changes in population seroprevalence across time (regardless of the actual number values) can be indicative of the effectiveness of MDA. As long as the method is fixed and replicable, then both longitudinal and between-population comparisons are appropriate and will have a fixed level of error, even though the absolute accuracy will remain unknown. The real value of using an internally controlled method such as FMM or EM is that it is possible to use an algorithmic approach that is simple to apply to any data set and which requires no additional testing of external specimens or controls. In this study, we generated a ROC curve based on specimens that had previously been calibrated against the original reference standards described by Goodhew *et al* [19]. There is no gold standard for serological testing of chlamydia, and mis-classification in the reference standards is likely to have introduced error in the reference panel. Goodhew described how one PCR-positive DBS tested negative for antibodies against Pgp3, while three samples that were in the negative reference group tested positive for antibodies against Pgp3 [19]. As these original reference standards were no longer available, we have had to rely on a second set

of standards that were tested against the original standards. Problems relating to the ROC reference specimens could be solved by the establishment of a fully maintained and quality controlled international standard, but this is unlikely to happen as it would be very difficult to identify a reliable source of large volumes of seropositive plasma.

FMM has been used in numerous serological studies [34,39,43,45,60–66] and we propose that it, or the closely related EM, should be considered as the method of choice when performing data analysis for trachoma serology data. In trachoma control programmes, the SD parameter should be adjusted to favour high specificity and a larger number of SDs than used here would seem appropriate. One attractive option would be to use data from a post-elimination country (i.e. the Gambia) to subtract out the background positivity and by doing so calibrate or normalise the test for use in populations where elimination has not yet been reached and prevalence is unknown.

Variability and error are inherent to any diagnostic test and with every change in reference standard and assay technique, variability and error increase over and above any variation that may be inherent in a test due to inter- or intra- centre and user variation. Thus, we believe that an alternate approach to assay design, reference selection and threshold specification should be considered.

For all the sample sets included in this study, the density data peak around 0.25 OD₄₅₀ (Figs 2A, 3A and 4A), suggesting that a comparison of seroprevalence levels between populations is possible. Compared to ROC curves, internally-referenced thresholds inherently account for differing background levels in each population. If not accounted for using the ROC curve, this may result in an under- or over-estimation of seroprevalence. This will facilitate the programmatic usage of seroprevalence levels set by the finite mixture model or expectation-maximisation algorithm if serology is to be adopted as an alternative monitoring method.

Conclusion

The ELISA assay presented in this paper is easy-to-use, affordable in terms of both reagents and equipment required, and can potentially be deployed in low- and middle-income countries. The unit cost per sample was less than £4.00; this includes all materials required for sample collection and DBS testing, including reagents, ELISA plates and sterile gloves. Our results show that the technological aspects of the assay are robust and that there is low variation both between replicate samples and plates and between populations, making it possible to compare seroprevalence levels between countries. Internally calibrated thresholding methods, such as the finite mixture model or the expectation-maximisation algorithm are more appropriate than thresholds set by a ROC curve, but for programmatic surveillance, they may require calibration using data from countries where trachoma has been declared as having been eliminated.

Supporting Information

S1 Table. Prevalence of the clinical signs of trachoma for Laos, by Gender, Region and Age.

(DOCX)

S2 Table. Prevalence of the clinical signs of trachoma for Uganda, by Gender, Region and Age.

(DOCX)

S3 Table. Prevalence of the clinical signs of trachoma for The Gambia by Gender, Region and Age.

(DOCX)

S4 Table. Seroprevalence for Laos by Gender, Region and Age, for each of six thresholds.
(DOCX)

S5 Table. Seroprevalence for Uganda by Gender, Region and Age, for each of six thresholds.
(DOCX)

S6 Table. Seroprevalence for The Gambia by Gender, Region and Age, for each of six thresholds.
(DOCX)

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Supplementary Table 4.1: Prevalence of the clinical signs of trachoma for Laos, by Gender, Region and Age.

	Prevalence of clinical signs (%)	
	N	TF
Overall	952	15 (1.6)
Female	423	9 (2.1)
Male	529	6 (1.1)
Attapu	406	11 (2.7)
Houaphan	307	3 (1.0)
Phôngsali	239	1 (0.4)
1 year old	78	2 (2.6)
2 years old	105	3 (2.9)
3 years old	101	5 (5.0)
4 years old	127	0
5 years old	114	1 (0.9)
6 years old	100	2 (2.0)
7 years old	100	1 (1.0)
8 years old	99	0
9 years old	128	1 (0.8)

TF = trachomatous inflammation, follicular

Supplementary Table 4.2: Prevalence of the clinical signs of trachoma for Uganda, by Gender, Region and Age

	Prevalence of clinical signs (%)		
	N	TF	TI
Overall	2700	93 (3.4)	8 (0.3)
Agogo	1353	43 (3.2)	1 (0.1)
Pader	1347	50 (3.7)	7 (0.5)
Female	1351	48 (3.6)	6 (0.4)
Male	1349	45 (3.6)	2 (0.1)
1 year old	242	11 (4.5)	2 (0.8)
2 years old	353	17 (4.8)	0
3 years old	358	16 (4.5)	3 (0.8)
4 years old	336	14 (4.2)	1 (0.3)
5 years old	329	12 (3.6)	1 (0.3)
6 years old	333	8 (2.4)	0
7 years old	264	6 (2.3)	1 (0.4)
8 years old	235	3 (1.3)	0
9 years old	250	6 (2.4)	0

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense

Supplementary Table 4.3: Prevalence of the clinical signs of trachoma for The Gambia by Gender, Region and Age

	Prevalence of clinical signs (%)					
	N	TF	TI	TS	TT	CO
Overall	1868	30 (1.6)	4 (0.2)	78 (4.2)	8 (0.4)	1 (0.1)
LRR	1028	18 (1.8)	4 (0.4)	55 (5.4)	7 (0.7)	1 (0.1)
URR	840	12 (1.4)	0	23 (2.7)	1 (0.1)	0
Female	1080	10 (0.9)	3 (0.3)	52 (4.8)	5 (0.5)	1 (0.1)
Male	788	20 (2.5)	1 (0.1)	26 (3.3)	3 (0.4)	0
<1 year old	36	0	0	0	0	0
1 year old	65	1 (1.5)	0	0	0	0
2 years old	88	3 (3.4)	0	2 (2.3)	0	0
3 years old	101	8 (7.9)	0	1 (1.0)	0	0
4 years old	96	5 (5.2)	0	1 (1.0)	0	0
5 years old	96	3 (3.1)	0	1 (1.0)	0	0
6 years old	89	0	0	2 (2.2)	0	0
7 years old	77	2 (2.6)	1 (1.3)	0	0	0
8 years old	78	1 (1.3)	0	0	0	0
9 years old	52	2 (3.8)	1 (1.9)	0	0	0
10-19	412	4 (1.0)	1 (0.2)	2 (0.5)	0	0
20-29	191	0	0	1 (0.5)	0	0
30-39	152	1 (0.7)	1 (0.7)	5 (3.3)	0	0
40-49	99	0	0	5 (5.1)	0	0
50-59	95	0	0	15 (15.8)	2 (2.1)	0
60+	141	0	0	43 (30.5)	6 (4.3)	1 (0.7)
1-9 year olds -LRR	383	14 (3.7)	2 (0.5)	1 (0.3)	0	0
1-9 year olds -URR	359	11 (3.1)	0	6 (1.7)	0	0
≥10 year olds-LRR	645	4 (0.6)	2 (0.3)	54 (8.45)	7 (1.1)	1 (0.2)
≥10 year olds-URR	481	1 (0.2)	0	17 (2.6)	1 (0.2)	0

TF = trichomatous inflammation, follicular; TI = trichomatous inflammation-intense; TS = trichomatous scarring; TT = trichomatous trichiasis; CO = corneal opacity

Supplementary Table 4.4: Seroprevalence for Laos by Gender, Region and Age, for each of six thresholds.

Threshold, % (95% confidence interval)													
		VIP		EM		FMM		ROC Youden's J-index		ROC Sensitivity>80%		ROC Specificity>98%	
	N	OD=0.619		OD=0.650		OD=0.696		OD=0.870		OD=0.965		OD=1.951	
Overall	952	11.3%	(9.4-13.6)	10.4%	(8.6-12.6)	15.6%	(13.3-18.0)	6.7%	(5.3-8.6)	6.3%	(4.9-8.1)	1.1%	(0.5-2.0)
Female	423	10.4%	(7.7-13.8)	9.9%	(7.3-13.3)	15.6%	(12.3-19.5)	6.9%	(4.7-9.8)	6.4%	(4.3-9.3)	0.70%	(0.2-2.2)
Male	529	12.1%	(9.5-15.3)	10.8%	(8.3-13.8)	15.5%	(12.6-18.9)	6.6%	(4.7-9.2)	6.2%	(4.4-8.7)	1.3%	(0.6-2.8)
Attapu	406	4.4%	(2.7-7.0)	3.7%	(2.2-6.2)	9.4%	(6.8-12.7)	0.70%	(0.2-2.3)	0.70%	(0.19-2.3)	0	(0-1.1)
Houaphan	307	21.5%	(17.1-26.6)	20.5%	(16.2-25.6)	24.1%	(19.5-29.4)	16.0%	(12.1-20.7)	15.0%	(11.3-19.6)	2.3%	(1.0-4.8)
Phôngsali	239	10.0%	(6.7-14.7)	8.8%	(5.7-13.3)	15.1%	(10.9-20.4)	5.0%	(2.7-8.8)	4.6%	(2.4-8.3)	1.3%	(0.3-3.9)
1 year old	78	10.2%	(4.8-19.7)	10.2%	(4.8-19.7)	10.2%	(4.8-19.7)	9.0%	(4.0-18.2)	9.0%	(4.0-18.2)	2.6%	(0.4-9.8)
2 years old	105	8.6%	(4.2-16.1)	8.6%	(4.2-16.1)	14.3%	(8.5-22.7)	6.7%	(3.0-13.7)	6.7%	(3.0-13.7)	0	(0-4.3)
3 years old	101	9.9%	(5.1-17.9)	8.9%	(4.4-16.7)	12.9%	(7.3-21.4)	6.9%	(3.1-14.2)	6.9%	(3.1-14.2)	2.0%	(0.3-7.7)
4 years old	127	13.4%	(8.2-20.8)	12.6%	(7.6-19.9)	15.7%	(10.1-23.5)	9.4%	(5.2-16.3)	9.4%	(5.2-16.3)	0.80%	(0.04-5.0)
5 years old	114	6.1%	(2.7-12.7)	4.4%	(1.6-10.4)	8.8%	(4.5-15.9)	2.6%	(0.7-8.1)	2.6%	(0.7-8.1)	0.90%	(0.04-5.5)

6 years old	100	12.0%	(6.6-20.4)	11.0%	(5.9-19.2)	18.0%	(11.3-27.2)	6.0%	(2.5-13.1)	6.0%	(2.5-13.1)	3.0%	(0.8-9.2)
7 years old	100	8.0%	(3.8-15.6)	8.0%	(3.8-15.6)	13.0%	(7.4-21.6)	6.0%	(2.5-13.1)	6.0%	(2.5-13.1)	0	(0-4.6)
8 years old	99	15.2%	(9.0-24.1)	12.1%	(6.7-20.6)	24.2%	(16.4-34.1)	8.1%	(3.8-15.8)	7.1%	(3.1-14.5)	1.0%	(0.-6.3)
9 years old	128	17.2%	(11.3-25.1)	16.4%	(10.7-24.2)	21.1%	(14.6-29.4)	6.35%	(2.9-12.3)	3.9%	(1.4-9.3)	0	(0-3.6)

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal opacity; VIP = visual inflection point; EM = expectation-maximisation algorithm; FMM = finite mixture model; OD = optical density, measured at 450 nm

Supplementary Table 4.5: Seroprevalence for Uganda by Gender, Region and Age, for each of six thresholds.

Threshold, % (95% confidence interval)													
		VIP		EM		FMM		ROC Youden's J- index		ROC Sensitivity>80%		ROC Specificity>98%	
	N	OD=0.641		OD=0.450		OD=0.554		OD=0.870		OD=0.965		OD=1.951	
Overall	2700	13.4%	(12.1- 4.7)	24.3%	(22.7-26.0)	17.1%	(16.0-18.9)	6.8%	(5.9-7.8)	5.3%	(4.5-6.2)	0.3%	(0.1-0.8)
Female	1351	13.3%	(11.6-15.3)	23.8%	(21.5-26.1)	17.2%	(15.7-19.1)	6.2%	(5.0-7.7)	5.0%	(3.9-6.3)	0.3%	(0.1-0.8)
Male	1349	13.4%	(11.7-15.4)	24.9%	(22.6-27.3)	17.8%	(15.8-20.0)	7.4%	(6.1-9.0)	5.6%	(4.5-7.0)	0.2%	(0.1-0.7)
Agogo	1353	12.6%	(10.9-14.6)	23.3%	(21.1-25.6)	15.9%	(14.0-18.0)	6.0%	(4.8-7.4)	4.9%	(3.8-6.2)	0.3%	(0.1-0.8)
Pader	1347	14.1%	(12.3-16.1)	25.4%	(23.1-27.8)	18.9%	(16.9-21.1)	7.7%	(6.3-9.2)	5.7%	(4.6-7.1)	0.2%	(0.1-0.7)
1 year old	247	2.4%	(1.0-5.5)	7.3%	(4.5-11.4)	3.6%	(1.8-7.0)	1.6%	(0.5-4.4)	0.8%	(0.1-3.2)	0	(0-1.9)
2 years old	365	5.5%	(3.5-8.5)	10.7%	(7.8-14.4)	7.9%	(5.5-11.3)	3.8%	(2.2-6.5)	3.8%	(2.2-6.5)	0.3%	(0-1.8)
3 years old	367	7.1%	(4.8-10.3)	18.3%	(14.5-22.7)	10.6%	(7.8-14.3)	3.5%	(2.0-6.1)	2.2%	(1.0-4.4)	0.3%	(0-1.8)
4 years old	349	11.5%	(8.4-15.4)	22.1%	(17.9-26.9)	15.2%	(11.7-19.5)	4.9%	(3.0-7.8)	4.0%	(2.3-6.8)	0	(0-1.4)
5 years old	331	15.7%	(12.1-20.2)	27.2%	(22.5-32.4)	18.4%	(14.5-23.1)	8.2%	(5.5-11.8)	6.6%	(4.3-10.0)	0.6%	(0.1-2.0)
6 years old	344	18.3%	(14.5-22.9)	31.1%	(26.3-36.3)	23.5%	(19.2-28.5)	8.1%	(5.6-11.7)	6.4%	(4.1-9.7)	0	(0-1.4)

7 years old	269	16.7%	(12.6-21.9)	32.3%	(26.9-38.3)	23.4%	(18.6-29.0)	9.3%	(6.2-13.6)	6.7%	(4.1-10.5)	0	(0-1.8)
8 years old	240	20.4%	(15.6-26.2)	32.5%	(26.7-38.9)	24.2%	(19.0-30.2)	9.2%	(6.0-13.7)	7.5%	(4.6-11.8)	0.8%	(0.1-3.3)
9 years old	253	23.7%	(18.7-29.5)	37.2%	(31.2-43.4)	30.4%	(24.9-36.6)	13.4%	(9.6-18.4)	9.9%	(6.6-14.4)	0.4%	(0-2.5)

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal

opacity; VIP = visual inflection point; EM = expectation-maximisation algorithm; FMM = finite mixture model; OD = optical density, measured at 450 nm

Supplementary Table 4.6: Seroprevalence for The Gambia by Gender, Region and Age, for each of six thresholds.

Threshold, % (95% confidence interval)													
		VIP		EM		FMM		ROC Youden's J-index		ROC Sensitivity>80%		ROC Specificity>98%	
	N	OD=0.570		OD=0.570		OD=0.672		OD=0.870		OD=0.965		OD=1.951	
Overall	1868	29.3%	(27.3-31.5)	29.3%	(27.3-31.5)	26.2%	(24.2-28.2)	20.9%	(19.1-22.9)	18.9%	(17.2-20.8)	3.3%	(2.6-4.3)
Female	1080	35.0%	(32.2-37.9)	35.0%	(32.2-37.9)	31.6%	(28.8-34.5)	26.0%	(23.4-28.8)	23.7%	(21.2-26.4)	4.8%	(3.7-6.3)
Male	788	21.6%	(18.8-24.6)	21.6%	(18.8-24.6)	18.7%	(16.1-21.7)	14.0%	(11.7-16.6)	12.3%	(10.1-14.9)	1.3%	(0.7-2.4)
LRR	1028	33.9%	(31.0-36.8)	33.8%	(31.0-36.8)	30.5%	(27.7-33.4)	25.1%	(22.5-27.9)	22.6%	(20.1-25.2)	3.7%	(2.7-5.1)
URR	840	23.8%	(21.0-26.9)	23.8%	(21.0-26.9)	21.0%	(18.3-23.9)	15.8%	(13.5-18.5)	14.4%	(12.1-17.0)	2.9%	(1.9-4.3)
<1 year	36	0	(0-12.0)	0	(0-12.0)	0	(0-12.0)	0	(0-12.0)	0	(0-12.0)	0	(0-12.0)
1 year old	65	4.6%	(1.2-13.8)	4.6%	(1.2-13.8)	3.1%	(0.5-11.6)	0	(0-6.9)	0	(0-6.9)	0	(0-6.9)
2 years old	88	4.5%	(1.5-11.9)	4.5%	(1.5-11.9)	2.3%	(0.4-8.7)	1.1%	(0-7.1)	0	(0-5.2)	0	(0-5.2)
3 years old	101	5.9%	(2.4-13.0)	5.9%	(2.4-13.0)	5.9%	(2.4-13.0)	2.0%	(0.3-7.7)	2.0%	(0.3-7.7)	0	(0-4.6)
4 years old	96	8.3%	(3.9-16.2)	8.3%	(3.9-16.2)	7.3%	(3.2-14.90)	4.2%	(1.3-10.9)	4.2%	(1.3-10.9)	0	(0-4.8)
5 years old	96	8.3%	(3.9-16.2)	8.3%	(3.9-16.2)	6.3%	(2.6-13.6)	3.1%	(0.8-9.5)	3.1%	(0.8-9.5)	0	(0-4.8)
6 years old	89	5.60%	(2.1-13.2)	5.6%	(2.1-13.2)	4.5%	(1.4-11.7)	4.5%	(1.4-11.7)	3.4%	(0.9-10.2)	0	(0-5.2)

7 years old	77	10.2%	(4.9-20.0)	10.4%	(4.9-20.0)	7.8%	(3.2-16.8)	2.6%	(0.5-9.9)	2.6%	(0.5-9.9)	0	(0-5.9)
8 years old	78	10.3%	(4.8-19.7)	10.3%	(4.8-19.7)	7.7%	(3.2-16.6)	6.4%	(2.4-15.0)	3.8%	(1.0-11.6)	0	(0-5.8)
9 years old	52	19.2%	(10.1-33.0)	19.2%	(10.1-33.0)	15.4%	(7.3-28.6)	9.6%	(3.6-21.8)	9.6%	(3.6-21.8)	1.9%	(0.1-11.6)
10-19	412	21.4%	(17.6-25.7)	21.4%	(17.6-25.7)	15.5%	(12.2-19.5)	11.1%	(8.4-14.7)	9.7%	(7.1-13.1)	1.7%	(0.7-3.6)
20-29	191	36.6%	(29.9-43.9)	36.6%	(29.9-43.9)	31.4%	(25.0-38.6)	23.0%	(17.4-29.8)	19.9%	(14.6-26.4)	3.7%	(1.6-7.7)
30-39	152	53.9%	(45.7-62.0)	53.9%	(45.7-62.0)	52.0%	(43.8-60.1)	46.1%	(38.0-54.3)	44.1%	(36.1-52.3)	5.9%	(2.9-11.3)
40-49	99	69.7%	(59.5-78.3)	69.7%	(59.5-78.3)	65.7%	(55.4-74.7)	56.6%	(46.2-66.4)	50.1%	(40.3-60.1)	12.1%	(6.7-20.6)
50-59	95	74.7%	(64.5-82.8)	74.7%	(64.5-82.8)	72.6%	(62.4-81.0)	62.1%	(51.5-71.7)	55.8%	(45.3-65.9)	8.4%	(4.0-16.4)
60+	141	75.9%	(67.8-82.5)	75.9%	(67.8-82.5)	73.8%	(65.6-80.6)	63.1%	(54.5-71.0)	58.2%	(49.5-66.3)	12.8%	(7.9-19.7)

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal

opacity; VIP = visual inflection point; EM = expectation-maximisation algorithm; FMM = finite mixture model; OD = optical density, measured at 450nm

Chapter 5- *Serology reflects a decline in the prevalence of trachoma in two regions of The Gambia*



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SECTION A – Student Details

Student	Stephanie Mighelsen
Principal Supervisor	David Mabey
Thesis Title	Chlamydia trachomatis serology as a means of monitoring intervention activities to eliminate trachoma as a public health problem

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	Nature Scientific Reports		
When was the work published?	2017		
If the work was published prior to registration for your research degree, give a brief rationale for its inclusion	NA		
Have you retained the copyright for the work?*	openaccess	Was the work subject to academic peer review?	yes

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Where is the work intended to be published?	
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Stage of publication	

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I collected and assayed the samples, led on the analysis, wrote and edited the paper and responded to reviewers
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Student Signature: _____

Date: 10/09/18

Supervisor Signature: _____

Date: 12/4/18

Summary

In the previous chapter, methods of setting thresholds to define seropositivity were explored. Thresholds set using finite mixture modelling (FMM) were selected going forward.

In this chapter age-specific seroprevalence in two populations in The Gambia are used to estimate changes in the force of infection (Fol) and seroconversion and seroreversion rates. A population-based cross-sectional survey was conducted in Lower River Region (LRR) and Upper River Region (URR).

One population (LRR) received MDA because the prevalence of TF was greater than 10% in children aged 1-9 years old; the other population (URR) did not receive MDA because the prevalence of TF was not above the 10% threshold. In the current survey, both regions had less than 5% TF in children aged 1-9 years old. Seroprevalence was measured using antibodies against Pgp3. Seroprevalence was significantly different in the two regions: 26.2% in LRR and 17.1% in URR. Age-specific seroprevalence was used with reverse catalytic modelling to show a decrease in the transmission of Ct infection in both regions over 15 years ago. This change in the force of infection likely reflects a secular decline as access to healthcare, sanitation and water has increased over the past 30 years, as well as a previously unpublished mass drug campaign with topical tetracycline. Seroprevalence data was also used to estimate changes in seroconversion and seroreversion, which showed a reduction in seroconversion over time in both populations.

The serological analysis support findings from clinical examination and test for infection but is confounded by the fact that the antibodies detected in this study are unable to distinguish between ocular and genital Ct infection, potentially confounding results as changes in seroconversion may be due to historical interventions against trachoma or may reflect more recent acquisition of genital Ct infection.

This chapter demonstrates a potential use for serological data beyond simple estimates of population seroprevalence, showing how the age-specific prevalence of antibodies can be used to demonstrate past changes in the force of infection. In the subsequent chapter antibody levels, rather than seroprevalence data, is used to model changes in the force of infection over time and between populations.

SCIENTIFIC REPORTS

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Serology reflects a decline in the prevalence of trachoma in two regions of The Gambia

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Trachoma is caused by *Chlamydia trachomatis* (Ct). It is targeted for global elimination as a public health problem. In 2014, a population-based cross-sectional study was performed in two previously trachoma-endemic areas of The Gambia. Participants of all ages from Lower River Region (LRR) (N = 1028) and Upper River Region (URR) (N = 840) underwent examination for trachoma and had blood collected for detection of antibodies against the Ct antigen Pgp3, by ELISA. Overall, 30 (1.6%) individuals had active trachoma; the prevalence in children aged 1–9 years was 3.4% (25/742) with no statistically significant difference in prevalence between the regions. There was a significant difference in overall seroprevalence by region: 26.2% in LRR and 17.1% in URR ($p < 0.0001$). In children 1–9 years old, seroprevalence was 4.4% in LRR and 3.9% in URR. Reversible catalytic models using information on age-specific seroprevalence demonstrated a decrease in the transmission of Ct infection in both regions, possibly reflecting the impact of improved access to water, health and sanitation as well as mass drug administration campaigns. Serological testing for antibodies to Ct antigens is potentially useful for trachoma programmes, but consideration should be given to the co-endemicity of sexually transmitted Ct infections.

Trachoma is caused by ocular infection with the obligate intracellular bacterium *Chlamydia trachomatis* (Ct). It is the leading infectious cause of blindness worldwide¹. Infection is associated with clinical signs of inflammation in the conjunctiva, known as active trachoma; these include trachomatous inflammation—follicular (TF) and trachomatous inflammation—intense (TI). Many repeated episodes of active trachoma over years to decades can lead to trachomatous trichiasis (TT), which may lead to impaired vision. The World Health Organization (WHO) estimates that over 200 million people in 42 countries are at risk of blindness from trachoma². In 2010, approximately 1.9 million people suffered from visual impairment or blindness due to trachoma¹. The WHO Alliance for the Global Elimination of Trachoma by 2020 (GET2020) aims to eliminate trachoma as a public health problem by 2020² through the SAFE Strategy (Surgery, Antimicrobials, Facial cleanliness and Environmental improvement). The WHO-endorsed strategy for global control is to reduce the population prevalence of TF to <5% in children aged 1–9 years and the prevalence of unmanaged TT to <0.2% in adults aged 15 years and above³. By 2014, seven countries had reported having met these targets nationally⁴. The Gambia has been a hub for trachoma research for over 50 years and is on course to declare the elimination of trachoma as a public health problem by 2020. Two National Surveys of Blindness and Low Vision in The Gambia demonstrated that there had been a nation-wide decrease in the prevalence of active trachoma and TT between 1986 and 2000^{5,6}. Specific elimination efforts, undertaken between 2007 and 2010 and run by the National Eye Health Programme (NEHP), included mass drug administration (MDA) of azithromycin in 23 districts across the country. The Partnership for the Rapid Elimination of Trachoma (PRET)⁷ was embedded within the national programme and measured the prevalence of Ct infection in children residing in four districts in which MDA had been administered. Deployment

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	Both Regions		Lower River Region		Upper River Region	
	N	%	N	%	N	%
Overall	1832		1010	55.1	822	44.9
Gender						
Female	1056	57.7	584	58.0	472	57.0
Male	776	42.2	426	42.0	350	43.0
Age group (years)						
1–9	742	39.7	383	37.3	359	42.7
10–19	412	22.1	231	22.5	181	21.5
20–29	191	10.2	101	9.8	90	10.7
30–39	152	8.1	79	7.7	73	8.7
40+	335	17.9	216	21.0	119	14.2

Table 1. Age distribution of study participants, Lower River Region and Upper River Region, The Gambia, 2014.

of interventions and disease control measures across The Gambia was not even, with some districts receiving the full SAFE intervention and others receiving reduced (F & E components) or no specifically targeted interventions. It is important to be able to evaluate the effectiveness of these interventions and a significantly reduced or halted transmission of infection is a key indicator that elimination may have been achieved.

The current guidelines for post-intervention surveillance of Ct transmission intensity are based on the prevalence of TF in children aged 1–9 years. This is problematic because in the peri-elimination period and low-endemicity settings^{7,8} the correlation between the clinical signs of trachoma and the presence of ocular Ct infection is poor. This diminishment of the positive predictive value of clinical signs means that the false positive rate of TF screening is increased and the specificity of the clinical sign is negatively affected.

Reduced global endemicity also leads to the unmasking of diseases that resemble trachoma clinically but which have no clear link to Ct infections. We recently surveyed the western division of the Solomon Islands⁹, a population where TF was not found to be a highly specific indicator of ocular Ct infection. Around 26% of 1–9 year olds living there had TF, but Ct infection was very scarce at just 1.3%. Using a serological tool we showed that 53/66 (80%) of the cases of TF that we observed were in people who were serologically negative for prior Ct infection¹⁰. Clinical signs of trachoma were also a poor indicator for the need to deploy antimicrobials in Fiji, where the high prevalence of TF cases could be better explained by socio-epidemiological practices of eyelash depilation than by Ct infections¹¹. The current WHO guidelines would recommend MDA in both Fiji and the Solomon Islands, but the evidence from more detailed surveys makes an argument against the likelihood that the use of antimicrobials would be effective. Situations such as this highlight the need to develop new tools that can support programmes to make informed decisions about how to use antimicrobials in trachoma control. Antibodies against *Chlamydia trachomatis* reflect cumulative exposure to Ct^{12,13} and it has been suggested that programmes could use some measure of seroprevalence as an alternative indicator of changes in transmission^{14,15}. Previous work has investigated the use of age-specific seroprevalence for surveillance in the peri-¹⁶ and post-MDA setting^{15,17,18}. Serological techniques for the detection of antibodies against Ct have been used to study the epidemiology of both urogenital and ocular Ct infections^{19,20}. An enzyme-linked immunoassay (ELISA) which detects antibodies against Pgp3 (pCT03) has recently been used for analysis of samples collected from trachoma-endemic regions^{21,22}. The Pgp3 molecule is an immunogenic Ct-specific protein that is encoded by the Ct plasmid and which is highly conserved at the genetic level among Ct isolates²³.

In the current study we have used serological data from a Pgp3-specific ELISA to gain insight into the dynamics of Ct transmission in two regions of The Gambia, one of which (the Lower River Region [LRR]) had received three annual rounds of MDA, whilst the other (the Upper River Region [URR]) had not. URR was not targeted for antimicrobial use because the TF prevalence had already dropped below the WHO threshold for elimination as a public health problem.

To explore the utility of serology as a tool for surveillance in trachoma elimination programmes, we have modelled the seroconversion rate (SCR) in URR and LRR under different epidemiological settings. The SCR is the yearly average rate by which seronegative individuals become seropositive because of disease exposure. SCR is a metric that acts as a surrogate measure for the underlying force of infection (FoI) and the age specific SCR can be used to model changes in FOI across time, potentially identifying periods in which there were substantial changes in the rate of transmission of Ct infection in the population.

Results

We recruited participants of all ages from LRR (n = 1028, 41.9% male) and URR (n = 840, 42.5% male). Ten participants were excluded from the study because they either declined to provide a blood sample (n = 1) or had incomplete examination data (n = 9). The median participant age was 13 years in LRR (range: 1–88, IQR 6–34) and 11 years in URR (range: 0–90, IQR 5–40). The proportion of participants by age group is shown in Table 1. There were significantly more females than males overall ($X^2 = 45.332$, $p < 0.0001$), which held true in both LRR ($X^2 = 26.483$, $p < 0.0001$) and URR ($X^2 = 18.601$, $p < 0.0001$) and is representative of Gambian demographics²⁴. Age distributions were approximately equal between the two regions, as seen in Table 1 ($X^2 = 27.703$, $p = 0.14$).

	Frequency of signs (%)					
	N	TF	TI	TS	TT	CO
Overall	1832	30 (1.6)	4 (0.2)	78 (4.3)	8 (0.4)	1 (0.1)
Region						
LRR	1010	18 (1.8)	4 (0.4)	55 (5.4)	7 (0.7)	1 (0.1)
URR	822	12 (1.4)	0	23 (2.8)	1 (0.1)	0
Gender						
Female	1056	10 (0.9)	3 (0.3)	52 (4.9)	5 (0.5)	1 (0.1)
Male	776	20 (2.5)	1 (0.1)	26 (3.4)	3 (0.4)	0
Age group (years)						
1–9	742	25 (3.4)	2 (0.3)	7 (1.1)	0	0
10–19	412	4 (1.0)	1 (0.2)	2 (0.5)	0	0
20–29	191	0	0	1 (0.5)	0	0
30–39	152	1 (0.7)	1 (0.7)	5 (3.3)	0	0
40+	335	0	0	63 (18.8)	8 (2.4)	1 (0.3)
1–9 year olds -LRR	383	14 (3.7)	2 (0.5)	1 (0.3)	0	0
1–9 year olds -URR	359	11 (3.1)	0	6 (1.7)	0	0
≥10 year olds -LRR	645	4 (0.6)	2 (0.3)	54 (8.4)	7 (1.1)	1 (0.2)
≥10 year olds -URR	481	1 (0.2)	0	17 (3.5)	1 (0.2)	0

Table 2. Frequency of signs of trachoma in study participants, Lower River Region and Upper River Region, The Gambia, 2014. TF = trachomatous inflammation—follicular; TI = trachomatous inflammation—intense; TS = trachomatous conjunctival scarring; TT = trachomatous trichiasis; CO = corneal opacity LRR = Lower River Region; URR = Upper River Region.

Clinical signs. Thirty cases of TF were found in total (1.6%, 95% CI = 1.1–2.3%), of which 25 were in children aged 1–9 years (3.4%, 95% CI = 2.2–4.9, Table 2). There were 78 cases of trachomatous conjunctival scarring (TS), of which 70 were in participants aged 15 years and above (8.3%, 95% CI = 6.6–10.5); eight cases of TT, all of which were in those aged 40 years and older (0.9%, 95% CI = 0.4–1.8); and one case of corneal opacity (CO), in a participant over 40 years of age (0.1%, 95% CI = 0.0–0.6) (Table 2). The prevalence of TS was significantly different ($X^2 = 7.7932$, $p = 0.005$) between LRR (5.4%, 95% CI = 4.1–7.1%) and URR (2.8%, 95% CI = 1.8–4.2%). The TF prevalence in children aged 1–9 years was not significantly different ($X^2 = 0.199$, $p = 0.66$) between LRR (3.7%, 95% CI = 2.1–6.2%) and URR (3.1%, 95% CI = 1.6–5.6%). The prevalence of TI, TT and CO in this population was too low for further statistical analysis.

Data from this study show very low prevalence of active trachoma in these regions. In children aged 1–9 years, only 4.2% had detectable antibodies against Pgp3. In both regions, the prevalence of TF in 1–9-year-olds was below the 5% threshold for elimination as a public health problem as specified by the WHO.

Antibody responses in the populations. The threshold for seropositivity was set using a finite mixture model²⁵ to classify the samples as seropositive or seronegative based on maximum likelihood methods. The threshold was set at the mean of the Gaussian distribution of the seronegative population plus four standard deviations, 0.810 OD_{450nm} to ensure high specificity. Previous studies using the Pgp3 ELISA have commonly used a threshold set as three standard deviations above the mean of the negative population (the 97.5% confidence interval)^{21,26}. Using that lower threshold resulted in the same qualitative conclusions being drawn (See Supplementary Information).

The seroprevalence of antibodies against Pgp3 for each region, by age and gender, is summarised in Table 3. The overall seroprevalence in LRR and URR was 26.2% (95% CI = 23.5–29.0) and 17.1% (95% CI = 14.7–19.9%), respectively. Adjusting for multiple comparisons, there was a significant difference in overall seroprevalence between the two regions ($X^2 = 20.72$, $p < 0.0001$). Figure 1 shows the seroprevalence by age group and region. In children 1–9 years old, seroprevalence was 4.4% in LRR and 3.9% in URR. As expected, the prevalence of anti-Pgp3 antibodies increased with age using the non-parametric test for trend (z-score = 23.35, $p < 0.0001$; $\alpha = 0.01$). The seroprevalence doubled between 10–19-year-olds and the next oldest age group, 20–29-year-olds, both in the two regions combined, and in each region. Across both regions, in study participants who had no signs of trachoma, seroprevalence was 21% (95% CI = 19–23). Of those who had active trachoma (TF and/or TI) ($n = 30$), 3% were seropositive (95% CI = 0.1–17) and of those who had scarring trachoma (TS and/or TT and/or CO in either eye; $n = 122$) 56% were seropositive (95% CI = 44–67).

The prevalence of antibodies was significantly higher in females than in males (z-score = 6.384, $p < 0.0001$). The same was true in each region (LRR: z-score = 4.881, $p < 0.0001$; URR: z-score = 4.114, $p < 0.0001$). When data were considered for each age group, the seropositivity difference between males and females was only significant for 10–19-year-olds (z-score = 2.667, $p = 0.0077$) and 30–39-year-olds (z-score = 0.2551, $p = 0.0107$). Seroprevalence by gender and age group is shown in Fig. 2.

	Both				Lower River Region				Upper River Region			
	N	n	%	95% CI	N	n	%	95% CI	N	n	%	95% CI
Overall	1832	412	22.5	20.2–24.0	1010	268	26.5	23.5–29.0	822	144	17.5	14.7–19.9
Gender												
Female	1056	295	27.9	24.8–30.2	584	190	32.5	28.8–36.5	472	105	22.2	18.6–26.3
Male	776	117	15.1	12.4–17.5	426	78	18.3	14.8–22.4	350	39	11.1	8.1–15.0
Age group (years)												
1–9	742	31	4.2	2.9–5.9	383	17	4.4	2.6–7.0	359	14	3.9	2.1–6.5
10–19	412	48	11.7	8.7–15.1	231	29	12.6	8.6–17.5	181	19	10.5	6.4–16.0
20–29	191	48	25.1	19.1–31.9	101	28	27.7	19.3–37.5	90	20	22.2	14.1–32.2
30–39	152	73	48.0	39.9–56.3	79	41	51.9	40.4–63.3	73	32	43.8	32.2–55.9
40+	335	212	63.3	57.9–68.5	216	153	70.8	64.3–76.8	119	59	49.6	40.3–58.9

Table 3. Seroprevalence of anti-Pgp3 antibodies by region, gender and age, Lower River Region and Upper River Region, The Gambia, 2014.

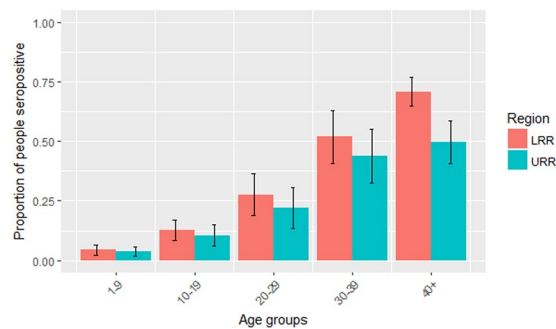


Figure 1. Proportion of participants who were seropositive for anti-Pgp3 antibodies by age group and region, Lower River Region (LRR) and Upper River Region (URR), The Gambia, 2014. Vertical bars indicate 95% CIs.

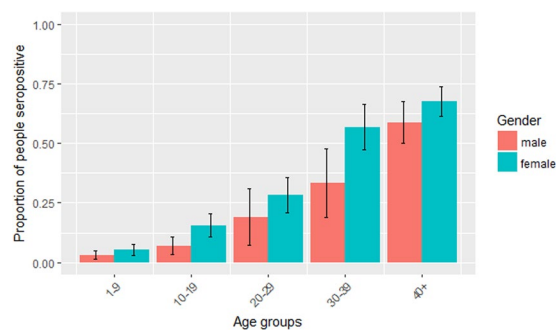


Figure 2. Proportion of participants who were seropositive for anti-Pgp3 antibodies, by age group and gender, Lower River Region and Upper River Region, The Gambia, 2014. Vertical bars indicate 95% confidence intervals.

Reduction in seroconversion rates over time. We used a reversible catalytic model together with the profile likelihood method to identify reductions in seroconversion rates (SCR) in both LRR and URR. The reversible catalytic model is based on the premise that individuals transit between seropositive and seronegative states with specific average rates²⁷. We compared three different models under which transmission dynamics might have changed across time. Our first model used the assumption that there had been a constant transmission, the second that there had at some point been an abrupt change in force of infection and the third that there had been a constant FOI to a point, after which it had decayed in a log-linear manner. We found that the model of abrupt change was most likely to explain the data we had observed in The Gambia. Abrupt reductions in SCRs were identified in both LRR and URR (Table 4 and Fig. 3) and these changes in SCR are estimated to have occurred respectively 23 and 16 years before data collection took place (Fig. 3). For LRR, the SCR would appear to have dropped from an incidence of 0.062 yearly events per person, to 0.010 yearly events per person. These estimates implied a putative 6.3-fold decrease in transmission intensity. In URR, the estimate of the past SCR dropped from

Region	SCR _{past}	SCR _{current}	SRR	Fold change
Lower River Region	0.095 (0.051–0.176)	0.015 (0.012–0.019)	0.008 (0.004–0.015)	6.3
Upper River Region	0.038 (0.018–0.082)	0.012 (0.009–0.016)	0.011 (0.003–0.039)	3.2

Table 4. Maximum likelihood estimates for the past and current seroconversion and seroreversion rates (SCR and SRR, respectively) associated with data collected from participants in Lower River Region and Upper River Region, The Gambia, 2014 where the respective 95% confidence intervals are shown in brackets.

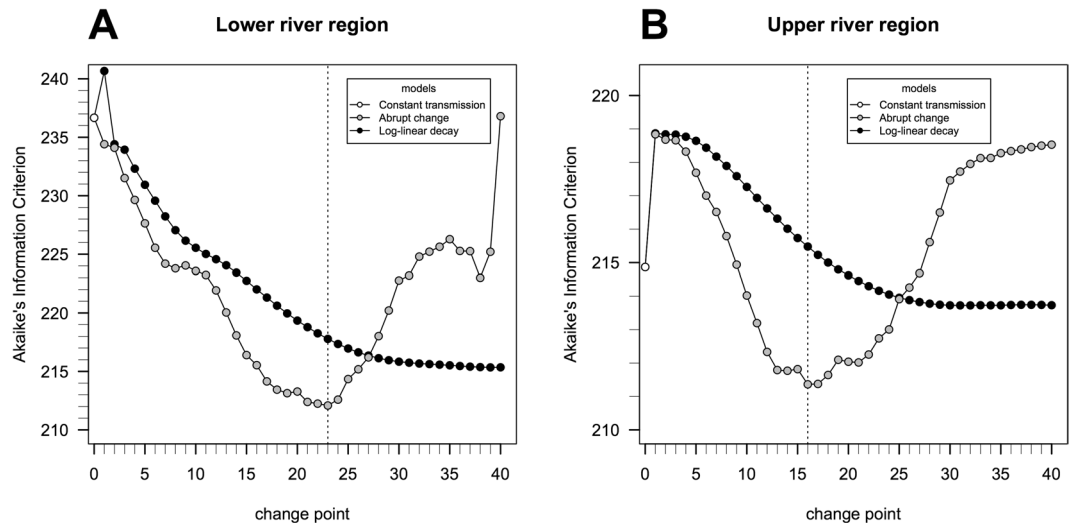


Figure 3. Akaike's information criterion (AIC) using the profile likelihood method for estimating the change-point for the models assuming an abrupt reduction in transmission intensity or annual log-linear decay of transmission intensity from the change-point to the present. In this analysis, the best model as function of change point is the one that leads to the minimum estimate of the AIC. Note that a change point of 0 is equivalent to the simple model assuming a constant transmission intensity over time. The results suggest abrupt reductions of transmission intensity 23 and 16 years before sampling for respectively the lower and upper river regions.

0.050 yearly events per person to 0.008 yearly events per person, a 3.2-fold decrease (Table 4). Figure 4 shows the expected seroprevalence curves as function of age assuming a change in transmission intensity.

Discussion

The Gambian government is currently compiling evidence for validation of trachoma elimination, following completion of its three-year TF/TT surveillance plan, which began in 2011^{28,29}. Previous studies have demonstrated declines in the prevalence of active trachoma in The Gambia both prior to^{8,30} and in response to the implementation of specific trachoma-control interventions^{31–33}. Increased access to water, education and healthcare in The Gambia during recent decades are thought to have had an impact³⁴, manifesting in a secular decline. Regardless of cause, the prevalence of active trachoma in 0–14-year-olds fell from 10.4% in 1986⁶ to less than 5% in 1996⁵ and has subsequently remained low. Prevalence data from historical studies is presented in Table 5. More recently, six years before the survey reported here, communities in LRR received azithromycin to treat ocular Ct infection, further reducing the prevalence of trachoma in this region^{7,29}, while communities in URR did not. Although not measured in our study, previous work has shown that there has been a reduction in the prevalence of ocular Ct infection in two villages in LRR^{35,36} with the most recent measurements showing 0.5% infection prevalence in PRET villages, a portion of which are in LRR⁷. In line with the findings of those previous studies, we provide a further data point showing TF prevalence <5% for each region. The prevalence of clinical signs strongly supports The Gambia's claim to have eliminated trachoma as a public health problem from these areas. The seroprevalence estimates and FoI modelling of trachoma transmission intensity offer further support that transmission is greatly reduced and that trachoma is no longer a public health problem in the areas studied. Seroprevalence data presented here are very similar to those seen in a recent study in Tanzania, where ocular Ct infection was eliminated in 2005³⁷ and serology shows an equally low prevalence of antibodies against Ct in children¹⁵.

We used three SCR models to estimate the change in SCR: the first model assumed no change in SCR and serves as a baseline for the second and third models, both of which did assume a change in SCR. The best model allowed for an acute change in SCR (Table 4, Fig. 3).

Our analysis of age-specific seroprevalence suggests that the FOI is currently very low, with a substantial decrease in SCR in children having occurred approximately 20 years ago (compared to the children that grew up before then). This change is too acute to reflect secular decline in the FOI of trachoma and may indicate the effects of interventions.

Region	Prevalence of signs of trachoma				
	1986 ⁶	1996 ⁶	TT (≥ 30 year-olds)	2013 ²⁹	TT (≥ 15 year-olds)
	TF/TI (0–14 year-olds)	TF/TI (0–9 year-olds)		TF (0–9 year-olds)	
Lower River Region	12.3%	11.5%	4.6%	1.8%	1.0%
Upper River Region	5.0%	1.3%	1.3%	0.4%	0.07%

Table 5. Previously published data on the prevalence active trachoma and trachomatous trichiasis, Lower River Region and Upper River Region, The Gambia, 1986–2013. Data from the 1986 survey was not available for 0–9 year olds, thus we have used the data for 0–14 year olds. TF = trachomatous inflammation - follicular, TI = trachomatous inflammation - intense, TT = trachomatous trichiasis.

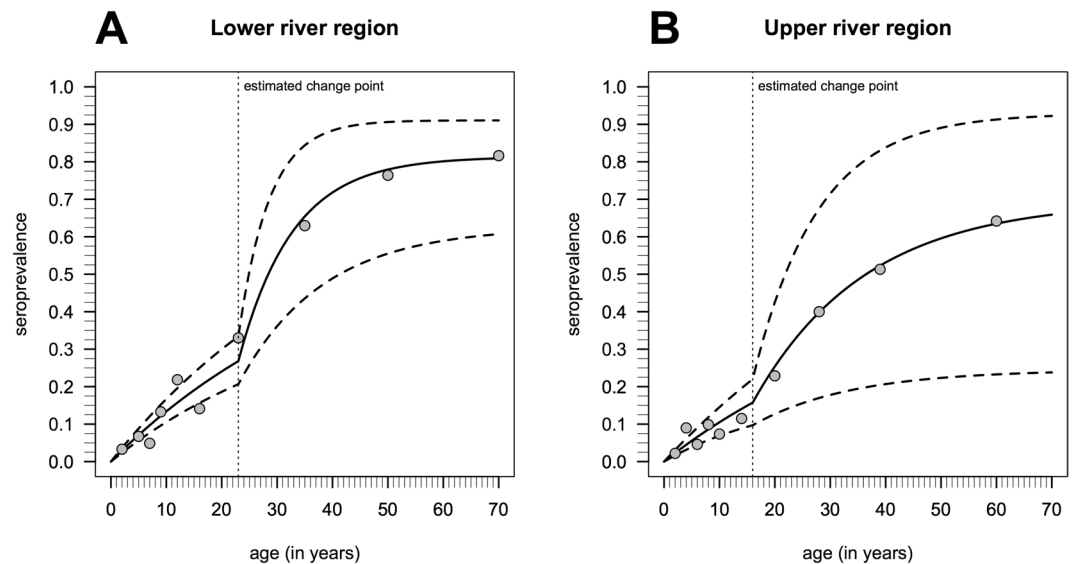


Figure 4. Expected seroprevalence curves as function of age (solid lines) according to the maximum likelihood estimates and the respective 95% confidence intervals (dashed lines) according to the best models selected in Fig. 3. The dots represent the observed seroprevalence when the age distribution was broken down in the respective deciles and the vertical pointed line refers to the change point estimated by the profile likelihood method.

One confounding factor in this analysis is that the serological test we used was not specific to ocular Ct infections; rather it indicates whether the individual being tested has antibodies against Ct that might originate from historical ocular or urogenital infections. Whilst the acute change in SCR that we identified would be consistent with a significant drop in the transmission intensity of ocular Ct in the mid to late 1990s, it could also be explained by a confounding signal from contemporary seroconversion events that relate to sexual activity of people in their late teens to mid-twenties. It is arguably most likely that the data reflect both things, but without current data describing the population prevalence of urogenital and ocular infections, the proportional contributions of STIs and trachoma to the change in SCR cannot be fully assessed.

No currently available serological test distinguishes between exposure to ocular and urogenital CT infection. It is interesting to note that the seroprevalence among 10–19-year-old Gambian females in our study was almost double that of their male counterparts of the same age. In the Gambia, the median age at which females first have sex is 18.6 years, with 52% of women aged 20–24 years surveyed as part of the Demographic and Health Survey (2013) having had sexual intercourse by age 20. In males the median age is 23.1 years, with 48% of men aged 25–29 years surveyed having had sexual intercourse by age 22³⁸. This could in part explain why we observed a gender difference in seroprevalence in the 10–19-year-old age range, as those with earlier sexual debut would be expected to be more likely to acquire STIs^{39–41} and to seroconvert.

In a population in which the urogenital Ct infection prevalence has been consistently low, it might be expected that anti-Pgp3 serological data would more accurately reflect longitudinal trends in ocular Ct transmission. Whilst data suggest that the prevalence of urogenital Ct infections has historically been very low in rural areas of The Gambia^{42,43}, there are no recent data, nor data based on modern molecular testing methods. A 2003 study from Malicounda in Thiès Region of neighbouring Senegal estimated that the prevalence of urogenital Ct infection there was just 0.3% ($n = 73$)⁴⁴. A systematic review of global estimates of incidence and prevalence of sexually transmitted infections (STI), including urogenital chlamydia, estimated that the prevalence of urogenital chlamydia was 2.9% in low-income countries⁴⁵, although it is noted that this study did not include data from The Gambia.

The cross-specificity of serological tests for antibodies emerging in response to ocular and urogenital Ct infections is a substantial hurdle that will need to be overcome if serological tests are to be widely deployed for trachoma monitoring.

The confidence intervals associated with SCRs seen in Fig. 4 are very broad (which in part reflects the uncertainty of modelling approaches) and comparison between the charts for LRR and URR is indicative but not conclusive of a difference in SCR between the two regions. Although a larger sample size would reduce the uncertainty, interpretation of the model depends to a large extent on the magnitude of the change as well as the timing between the change in SCR and sample collection, as seen in malaria modelling work²⁵. The very large sample sizes required of studies that could delineate SCR changes with high precision could be prohibitive. The analysis is further limited by the fact that the model assumes a memoryless property over time and the SCR models are defined as a function of the seroreversion rate, which is not easily estimated from a single cross-sectional survey.

A recent serological study using samples from Tanzania¹⁵ examined the age-specific seroprevalence of anti-Pgp3 antibodies in a trachoma-endemic community that had received two rounds of high coverage azithromycin MDA. The all-ages prevalence of ocular Ct infection had fallen from 9.5% to 0.1% two years after MDA⁴⁶, and to 0% five years after MDA³⁷, with a corresponding 11-fold decrease in SCR¹⁵. This change in infection prevalence occurred in a more defined (and probably narrower) timeframe than the one we have studied in The Gambia. This resulted in a more acute change in SCR, as has been demonstrated in malaria modelling exercises^{26,47}. Previous trachoma modelling studies suggest that an individual may require upwards of 100 lifetime ocular Ct infections in order to develop TT⁴⁸, so even a modest reduction in transmission may have significant public health implications and reduce the future incidence of TT.

Research is ongoing to address remaining challenges in interpreting trachoma seroprevalence. It is unclear how many infections are required for seroconversion to occur. Studies involving urogenital Ct infection suggest that just 68% of infected women produce IgG antibodies against Ct⁴⁹. The intensity of the inflammatory response, and the surface area of inflamed mucosa, however, are both likely to differ between the infected conjunctiva and infected female urogenital tract. Additionally, further work is needed to determine the half-life of Pgp3 antibodies and seroreversion rates. A previous study that examined a high-prevalence community before and after one round of azithromycin suggested that individual anti-Pgp3 antibody levels decreased slightly six months after drug treatment, but not enough to be considered seroreversion⁵⁰. This is similar to results seen for urogenital Ct infection, where anti-Pgp3 antibody titres decreased over 4–7 years, but patients were still in the seropositive range⁵¹. Although we have estimated SRR in this study (Table 4), a more accurate estimate could be obtained from a longitudinal study collecting serum samples over a period of years. In low transmission settings, such as post-MDA communities, the SRR may be under-estimated if the assumption of a balance between total number of seropositive and seronegative individuals does not hold true. Further studies to determine SRR are currently underway.

Dichotomising antibody levels to a simple seropositive/seronegative classification provides a straightforward estimation of seroprevalence, but SCR estimates could potentially be improved by using a model based on antibody levels and multiple sampling time points, as suggested by Yman *et al.*⁵². Such models might assume that antibody levels increase with age, as exposure is age-dependent and that transmission intensity can be calculated by measuring the boost in antibody levels. The use of age group-specific geometric mean antibody levels could be explored⁵² in addition to SCR.

Approximately 4% of 1–9-year-olds were positive for antibodies to Pgp3, which may be due to previously acquired ocular Ct infections and/or to ocular or respiratory Ct infections acquired at birth from mothers with urogenital Ct infections⁵³. This seroprevalence is within the range of prevalence values previously estimated in post-MDA surveys in Tanzania and Nepal^{14,15}. There was also no observed increase in anti-Pgp3 antibody positivity with age in 1–9-year-olds (Supplementary Table 2), in contrast to what is observed in trachoma-endemic settings, whether treatment-naïve¹⁸ or after 3 rounds of MDA^{17,21} and is in stark contrast to communities with a high prevalence of ocular infection¹⁶. Focusing on age-specific changes in seropositivity as a measure of cumulative exposure to ocular Ct infection might offset antibody responses from peri-natal infection, as the latter would be expected to be consistent across all ages, or even to decline with increasing age. The data from The Gambia presented here, combined with those from a variety of pre- and post-MDA settings, contribute to an understanding of the potential use of antibody-based surveillance of children to ensure a lack of infection recrudescence.

Data from paired pre- and post-MDA surveys, or longitudinal data from MDA surveys, would substantially improve parameters and modeling efforts. A detailed series of surveys in one population would help to develop generalized models for use elsewhere. The inclusion of infection data for both ocular and urogenital Ct infection is needed to further clarify how urogenital Ct contributes to observed seropositivity rates.

Methods

Ethical Review. This study was conducted in accordance with the Declaration of Helsinki. It received approval from the London School of Hygiene & Tropical Medicine Ethics Committee (LSHTM; reference 7059) and The Gambia Government/Medical Research Council Joint Ethics Committee (SCC1408v2). CDC investigators did not engage with study participants.

Survey methodology. We conducted a population-based, cluster-random-sampled survey was conducted in February–March 2014. The Gambia is divided into geographically-defined census Enumeration Areas (EAs) of approximately 600–800 people each. Sampling by EA is equivalent to sampling settlements with probability proportional to their size⁵⁴. Twenty EAs in each of URR and LRR were randomly selected for participation. Trained field workers sensitised villagers and obtained verbal community-consent from each village chief (*alkalo*). Field workers and *alkalos* compiled a list of households for each EA, from which households were randomly selected for census and recruitment. The study and consent form were explained to the head of each selected household

and prospective participants. All members of selected households were invited to participate, regardless of age. Written (thumbprint or signature) consent was obtained from each participant aged ≥ 18 years, while a parent or guardian provided written consent for each participating child aged under 18 years. Children aged 12–17 years provided assent before participating.

The trachoma graders were experienced in field grading for active trachoma and had received regular training according to PRET⁷ and Global Trachoma Mapping Project (GTMP) protocols^{55,56}. After informed consent was received, the grader examined both eyes of the subject using a binocular loupe (2.5 \times) and a torch. The grader changed gloves between each participant to minimise the risk of carry-over infection. In accordance with the Gambian NEHP policy, antibiotics were provided to individuals with evidence of active trachoma and to residents of their household.

Each participant had a finger-prick blood sample collected onto filter paper (Trop-Bio, Townsville, Australia) using a sterile single-use lancet (BD Microtrainer, Dublin, Ireland). Each filter paper had six extensions, calibrated to absorb 10 μ L of blood each. Samples were air-dried for approximately five hours and then placed in individual Whirl-Pak plastic bags (Nasco, Modesto, California) which were stored with desiccant sachets (Whatman, Little Chalfont, UK) at -20°C . All samples were shipped to LSHTM for testing.

ELISA Assay. Dried blood spots (DBS) were tested for antibodies against Pgp3 according to the method previously described²¹. Briefly, serum was eluted from dried blood spots then applied to a plate coated with Pgp3 protein¹⁷; known standards were included and assayed in triplicate on each plate. Following incubation, bound antibody was detected with HRP-labelled mouse anti-human IgG(Fc)-HRP (Southern Biotech, Birmingham, USA). Plates were incubated and washed, and then TMB (KPL, Gaithersburg, USA) was added to develop the plates. The reaction was stopped with 1 N H_2SO_4 and optical density was read at 450 nm (OD_{450}) on a Spectramax M3 plate reader (Molecular Devices, Wokingham UK). Readings were corrected for background by subtracting the average absorbance of three blank wells containing no serum, using Softmax Pro5 software (Molecular Devices).

Statistical Methods. Blanked OD_{450} values for samples were normalised against the 200 U standard included on each plate²¹. Laboratory work was undertaken masked to demographic and clinical information. Statistical analyses were carried out using R⁵⁷. Using the “survey” package and assuming a design effect of 2.65⁵⁵, the 95% confidence intervals (CI) were calculated using the Clopper-Pearson interval⁵⁸. Wilcoxon-Mann-Whitney z-scores⁵⁹ were calculated to compare the proportion seropositive between different regions, ages, and genders. The non-parametric test for trend was used to measure the increase in prevalence of anti-Pgp3 antibodies with age.

A finite mixture model²⁵ was used to classify the samples as seropositive or seronegative based on normalised OD_{450} values. The data were fitted using maximum likelihood methods, estimating the distribution parameters for each classification group (assumed seropositive or assumed seronegative) as well as the proportion of samples in each category to fit the overall distribution of results⁶⁰. To ensure that the assay had high specificity, the threshold for seropositivity was set using the mean of the Gaussian distribution of the seronegative population plus four standard deviations (the quantile inclusive of 99.994%) of the seronegative population^{25,60}.

Population age groups were categorized according to known time points of changes in disease prevalence. The youngest age group was 1–9-year-olds, who in LRR are likely to have been born during or after MDA with azithromycin. The oldest group included people aged 40 years and above, who experienced secular declines in trachoma prevalence prior to 1986, the year of the first National Survey of Blindness and Low Vision⁶. Participants aged between 10 and 39 years were grouped into 10-year categories.

Different reversible catalytic models were applied to the analysis of the data from each region. These models are described as function of seroconversion and seroreversion rates (SCR and SRR, respectively). SCR is defined as the annual mean rate by which seronegative individuals become seropositive upon disease exposure and is usually considered as a proxy of the transmission intensity of the population. SRR describes the annual mean rate by which seropositive individuals revert to a seronegative status in the absence of re-infection. Three reversible catalytic models were fit to the serological data of each region: (i) a simple model that assumes a constant SCR (i.e., transmission intensity) over time and people are born seronegative²⁷; (ii) a model with a constant historical SCR until a certain change point in the past where SCR changes abruptly to a new value (current SCR); (iii) a model that also assumes a historical seroconversion rate until a certain change point followed by a time period where SCR decays log-linearly every year. The respective parameter estimation was done using maximum likelihood principles. In particular, the models assuming a change point were estimated via profile likelihood method, as described elsewhere²⁵. Model comparison for the data of each region was based on the Akaike’s information criterion where the best model (and change point) is the one that provides the minimum estimate of this statistics.

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Author Contributions

S.J.M., R.B., D.C.W.M., A.W.S. and C.h.R. designed the study and wrote the paper, D.L.M., G.C., S.G. C.h.R. and S.J.M. developed the protocol for serological analysis, S.J.M. performed serological analysis, S.J.M., C.h.R. and N.S. performed the statistical analysis, R.B. trained the trachoma graders, S.J.M., H.J., P.M., R.B., S.E.B., D.C.W.M. and C.h.R. organized field data collection. H.J. and P.M. performed the field grading of trachoma. H.P. provided critical feedback on the paper. All authors reviewed and approved the manuscript.

Additional Information

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Competing Interests: S.G. is employed by the commercial company IHRC, Inc. and is a contractor at the Centres for Disease Control and Prevention. The authors have declared that no competing interests exist.

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SUPPLEMENTARY INFORMATION

We provide a more detailed description of the prevalence of clinical signs by age and gender in Supplementary Table 1, including prevalence in one-year increments for children 9 years and under, as well as extending the age groups up to 60+ years of age.

Supplementary Table 5.1. Clinical signs assessed in participants in The Gambia

	Prevalence of clinical signs (%)					
	N	TF	TI	TS	TT	CO
Overall	1832	30 (1.6)	4 (0.2)	78 (4.3)	8 (0.4)	1 (0.1)
LRR	1010	18 (1.8)	4 (0.4)	55 (5.4)	7 (0.7)	1 (0.1)
URR	822	12 (1.5)	0	23 (2.8)	1 (0.1)	0
Female	1057	10 (0.9)	3 (0.3)	52 (4.9)	5 (0.5)	1 (0.1)
Male	776	20 (2.6)	1 (0.1)	26 (3.4)	3 (0.4)	0
1 year old	65	1 (1.5)	0	0	0	0
2 years old	88	3 (3.4)	0	2 (2.3)	0	0
3 years old	101	8 (7.9)	0	1 (1.0)	0	0
4 years old	96	5 (5.2)	0	1 (1.0)	0	0
5 years old	96	3 (3.1)	0	1 (1.0)	0	0
6 years old	89	0	0	2 (2.2)	0	0
7 years old	77	2 (2.6)	1 (1.3)	0	0	0
8 years old	78	1 (1.3)	0	0	0	0
9 years old	52	2 (3.8)	1 (1.9)	0	0	0
10-19	412	4 (1.0)	1 (0.2)	2 (0.5)	0	0
20-29	191	0	0	1 (0.5)	0	0
30-39	152	1 (0.7)	1 (0.7)	5 (3.3)	0	0
40-49	99	0	0	5 (5.1)	0	0
50-59	95	0	0	15 (15.8)	2 (2.1)	0
60+	141	0	0	43 (30.5)	6 (4.3)	1 (0.7)
1-9 year olds -LRR	383	14 (3.7)	2 (0.5)	1 (0.3)	0	0
1-9 year olds -URR	359	11 (3.1)	0	6 (1.7)	0	0
≥10 year olds-LRR	627	4 (0.6)	2 (0.3)	54 (8.6)	7 (1.1)	1 (0.2)
≥10 year olds-URR	463	1 (0.2)	0	17 (3.7)	1 (0.2)	0

TF = trachomatous inflammation, follicular; TI = trachomatous inflammation-intense; TS = trachomatous scarring; TT = trachomatous trichiasis; CO = corneal opacity

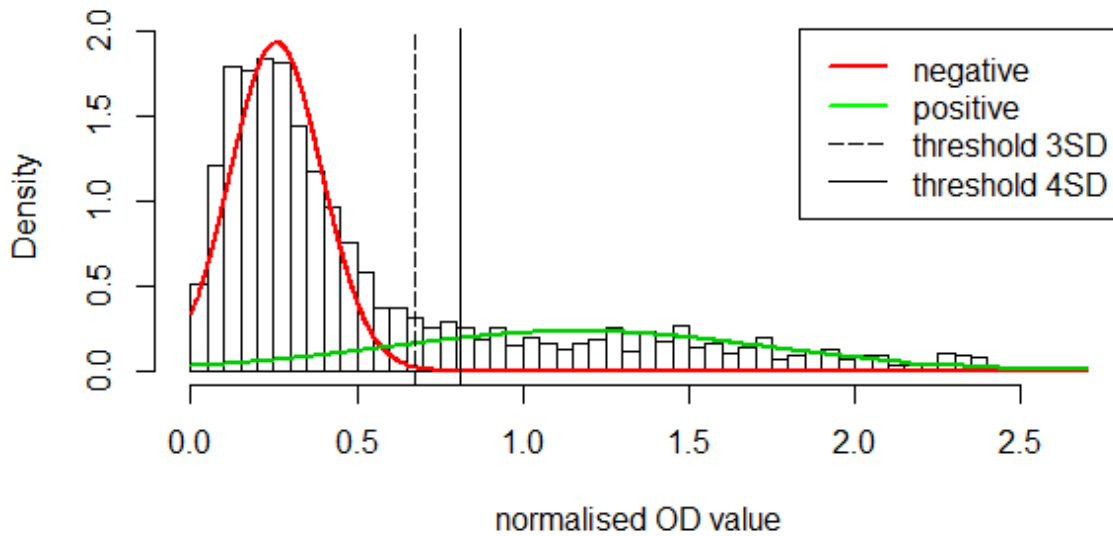
LRR = Lower River Region; URR = Upper River Region

Similarly, we provide a more detailed description of the seroprevalence of anti-Pgp3 antibodies in LRR and URR by region, gender and age in Supplementary Table 2. We again provide the seroprevalence in one-year increments for children 9 years and under, to facilitate comparison with other seroprevalence studies.

Supplementary Table 5.2. Seroprevalence of anti-Pgp3 antibodies in The Gambia by region, gender and age

	% seropositive (95% confidence interval)											
	Overall				Lower River Region				Upper River Region			
	N	n	%	95%CI	N	n	%	95%CI	N	n	%	95% CI
Overall	1868	489	26.18%	(24.2-28.2)	1028	313	30.45%	(27.7-33.4)	840	176	20.95%	(18.3-23.9)
Female	1080	341	31.57%	(28.8-34.5)	597	215	36.01%	(32.2-40.0)	483	126	26.09%	(22.2-30.2)
Male	788	148	18.78%	(16.1-21.7)	431	98	22.74%	(18.9-27.0)	357	50	14.01%	(10.7-18.1)
1 year old	65	2	3.08%	(0.5-11.6)	29	0	0	(0-1.9)	36	2	5.56%	(1.0-20.0)
2 years old	88	2	2.27%	(0.4-8.7)	33	2	6.06%	(1.1-21.6)	55	0	0	(0-8.1)
3 years old	101	6	5.94%	(2.4-13.0)	59	2	3.39%	(0.6-12.7)	42	4	9.52%	(3.1-23.5)
4 years old	96	7	7.29%	(3.2-14.90)	46	3	6.50%	(1.7-18.9)	50	4	8.00%	(2.6-20.1)
5 years old	96	6	6.25%	(2.6-13.6)	58	4	6.90%	(2.2-17.5)	38	2	5.26%	(1.0-19.1)
6 years old	89	4	4.49%	(1.4-11.7)	40	2	5.00%	(0.9-18.2)	49	2	4.08%	(0.7-15.1)
7 years old	77	6	7.79%	(3.2-16.8)	42	2	4.80%	(0.8-17.4)	35	4	11.43%	(3.7-27.7)
8 years old	78	6	7.69%	(3.2-16.6)	41	3	7.30%	(1.9-21.0)	37	3	8.11%	(2.1-23.0)
9 years old	52	8	15.38%	(7.3-28.6)	35	7	20.00%	(9.1-37.5)	17	1	5.88%	(0.3-30.8)
1-9	742	47	6.33%	(4.5-8.0)	383	25	6.53%	(3.9-8.9)	359	22	6.13%	(3.8-8.8)
10-19	412	65	15.78%	(12.2-19.5)	231	40	17.32%	(12.8-23.0)	181	24	13.26%	(8.8-19.3)
20-29	191	60	31.41%	(25.0-38.6)	101	35	34.65%	(25.6-44.8)	90	25	27.78%	(19.1-38.4)
30-39	152	79	51.97%	(43.8-60.1)	79	44	55.70%	(44.1-66.7)	73	35	47.95%	(36.2-59.9)
40-49	99	65	65.66%	(55.4-74.7)	62	47	75.81%	(63.0-85.4)	37	18	48.65%	(32.2-65.3)
50-59	95	69	72.63%	(62.4-81.0)	59	46	77.97%	(64.9-87.3)	36	23	63.89%	(46.2-78.7)
60+	141	104	73.76%	(65.6-80.6)	95	75	78.95%	(69.1-86.4)	46	29	63.04%	(47.5-76.4)

In our paper, we set the threshold for positivity at the mean of the Gaussian distribution of the seronegative population plus four standard deviations, 0.810 OD_{450nm}. Previous studies using the Pgp3 ELISA have commonly used a threshold set as three standard deviations above the mean of the negative population (the 97.5% confidence interval)^{15,20,21}. Supplementary Figure 1 shows the positive and negative populations with both thresholds.



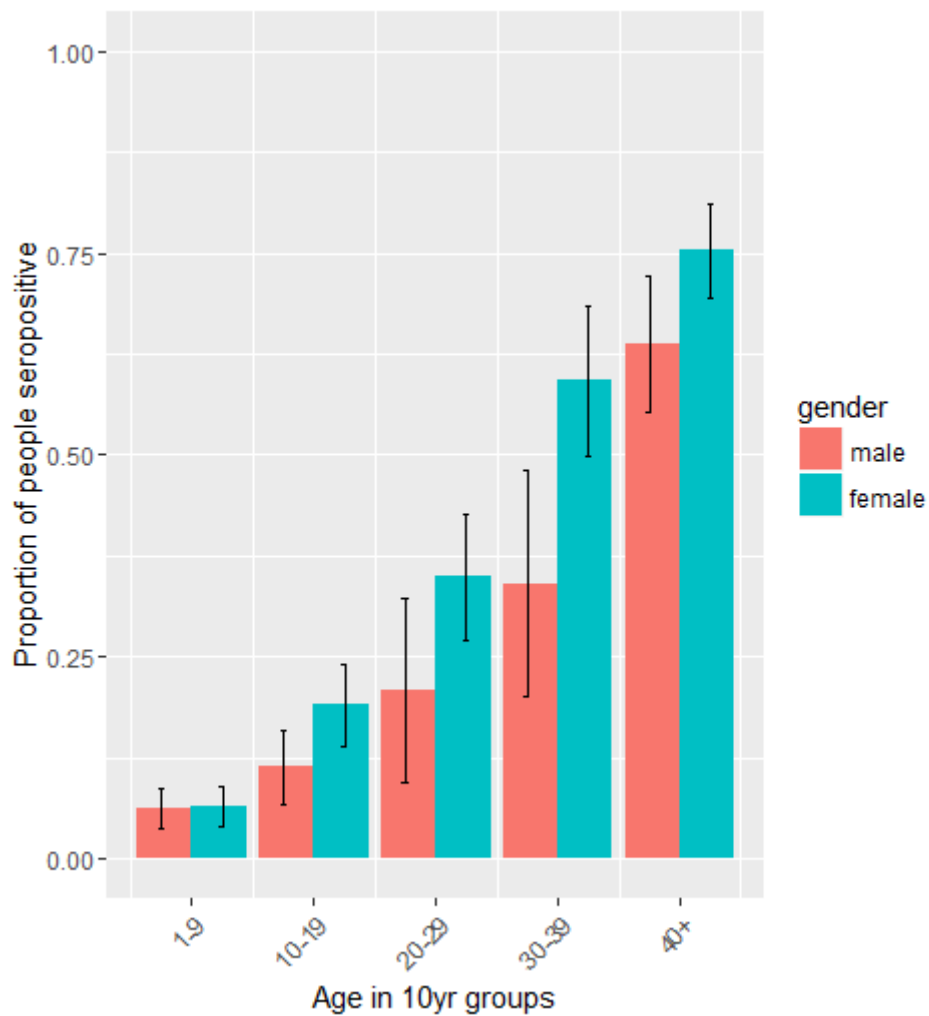
Supplementary Figure 5.1. Assumed seronegative (red) and assumed seropositive (green) populations as estimated using Finite Mixture Modelling.

The dashed line shows the threshold set using the mean of the negative population plus three standard deviations, while the solid line shows the more specific threshold set using the mean of the negative population plus four standard deviations.

Below we present the results had we used the typical threshold set using the mean of the Gaussian distribution of the seronegative population plus three standard deviations, 0.673 OD_{450nm}. Note that the estimated time change in seroconversion rates does not change.

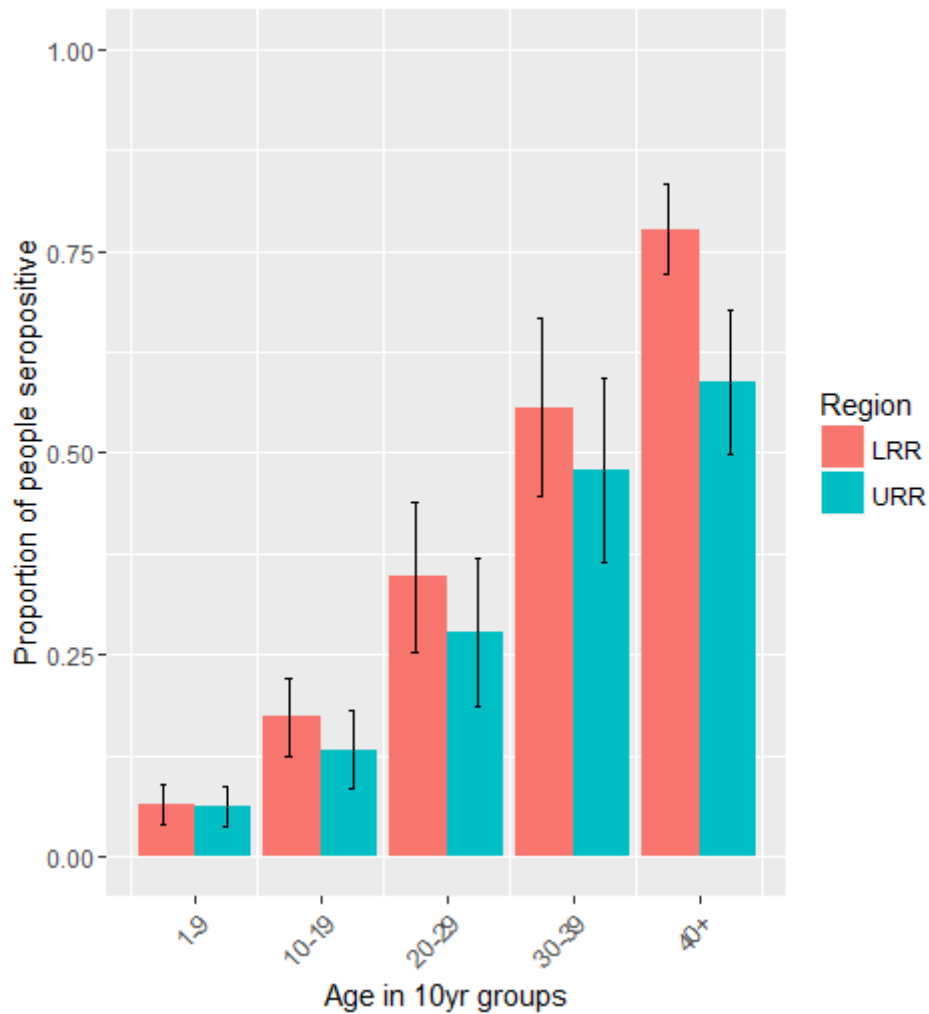
Supplementary Table 5.3. Seroprevalence of anti-Pgp3 antibodies by region, gender and age, Lower River Region and Upper River Region, The Gambia, 2014.

	Both regions combined			Lower River Region			Upper River Region		
	N	%	95%CI	N	%	95%CI	N	%	95% CI
Overall	1868	26.2	(24.2-28.2)	1028	30.5	(27.7-33.4)	840	20.9	(18.3-23.9)
Gender									
Female	1080	31.6	(28.8-34.5)	597	36.0	(32.2-40.0)	483	26.1	(22.2-30.2)
Male	788	18.8	(16.1-21.7)	431	22.7	(18.9-27.0)	357	14.0	(10.7-18.1)
Age (years)									
1-9	742	6.3	(4.5-8.0)	383	6.5	(3.9-8.9)	359	6.1	(3.8-8.8)
10-19	412	15.8	(12.2-19.5)	231	17.3	(12.8-23.0)	181	13.3	(8.8-19.3)
20-29	191	31.4	(25.0-38.6)	101	34.6	(25.6-44.8)	90	27.8	(19.1-38.4)
30-39	152	51.9	(43.8-60.1)	79	55.7	(44.1-66.7)	73	47.9	(36.2-59.9)
40+	335	71.0	(65.8-75.8)	216	77.8	(71.5-83.0)	119	35.2	(28.6-42.3)



Supplementary Figure 5.2. Proportion of participants who were seropositive for anti-Pgp3 antibodies using a threshold set at 0.673 OD450nm, by age group and region, Lower River Region (LRR) and Upper River Region (URR), The Gambia, 2014.

Vertical bars indicate 95% CIs.



Supplementary Figure 5.3. Proportion of participants who were seropositive for anti-Pgp3 antibodies using a threshold set at 0.673 OD_{450nm}, by age group and gender, Lower River Region and Upper River Region, The Gambia, 2014. Vertical bars indicate 95% confidence intervals.

Supplementary Table 5.4. Maximum likelihood parameter estimates and the respective 95% confidence intervals (in brackets) for the seroconversion and seroreversion rates (SCR and SRR, respectively); data collected from participants in Lower River Region and Upper River Region, The Gambia, 2014.

P-values <0.05 are indicative of a change in transmission intensity when comparing two reversible catalytic models, one assuming constant and stable transmission over and another assuming a sudden reduction in transmission intensity somewhere in the past.

	SCR _{past}	SCR _{current}	SRR	Fold change	p-value
Region					
Lower River Region	0.062 (0.038, 0.100)	0.014 (0.011, 0.018)	0.006 (0.003, 0.014)	4.4	<0.001
Upper River Region	0.038 (0.017, 0.082)	0.011 (0.009, 0.016)	0.011 (0.003, 0.039)	3.5	<0.001

Chapter 6- *Analysis of age-dependent mean antibody levels is more informative than qualitative sero-status analysis for studies on elimination of trachoma.*



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SECTION A – Student Details

Student	Stephanie Mighelsen
Principal Supervisor	David Mabey
Thesis Title	Chlamydia trachomatis serology as a means of monitoring intervention activities to eliminate trachoma as a public health problem

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

Where was the work published?	
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SECTION C – Prepared for publication, but not yet published

Where is the work intended to be published?	PLOS NTD
Please list the paper's authors in the intended authorship order:	Mighelsen, Martin, Massae, Dean, Bid, Sokana, Tekeraoi, Solomon, Mabey, Roberts
Stage of publication	ready to be submitted

SECTION D – Multi-authored work

For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	I collected samples from the Gambia, assayed samples from the Gambia, Laos & Uganda. I analysed all the samples, conducted the statistical analysis, wrote and edited the paper.
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Student Signature: _____

Date: 10/09/18

Supervisor Signature: _____

Date: 12/9/18

Summary

The previous chapters have explored setting seropositivity thresholds and estimating changes in seroprevalence. These analyses have relied on dichotomising the continuous data generated from ELISA and MBA assays.

This chapter explores the use of antibody levels, rather than seroprevalence, to monitor effects of MDA for trachoma elimination and to compare levels between populations, including those where trachoma has been eliminated as a public health problem and where MDA is ongoing.

In the following analyses, semi-quantitative antibody levels were used to create age-dependent geometric mean antibody level curves. This secondary analysis of antibody level data used a novel R package, 'tmleAb', which uses an ensemble machine learning approach to generate age-dependent antibody curves. These were compared between pre- and post-MDA communities of all ages in Nepal and a post-MDA community in Tanzania. Additionally, curves were compared for the two Gambian populations of all ages previously described in this thesis. Part of the analysis examined age-dependent curves for children from four countries between the ages of 1-9 years as this is the age range of interest in measuring the prevalence of clinical signs of trachoma. The final comparison was between the Gambian population, where there is a low prevalence of both ocular and genital Ct infection, and the Solomon Islands, where the prevalence of genital Ct infection is much higher which has affected the level of antibodies in the youngest children of the population.

Using antibody levels removes the need to establish the somewhat arbitrary thresholds between positive and negative samples and uses the 'raw' antibody level data directly. These analyses are more sensitive to changes in antibody levels and capture information as levels change that may not be otherwise captured if the change were too small to cross the positivity threshold. Additionally, the analysis in this chapter shows that at a population-level, wider confidence intervals are seen when a population is under the greatest force of Ct infection.

Title

Analysis of age-dependent mean antibody levels is more informative than qualitative sero-status analysis for studies on elimination of trachoma.

Authors

Stephanie J Migchelsen, Diana L Martin, Patrick A Massae, Deborah Dean, Rhiannon Bid, Oliver Sokana, Rabebe Tekeraoi, Anthony W Solomon, David C W Mabey, Chrissy h Roberts

6.1 Abstract

Background

Detecting antibodies against *Chlamydia trachomatis* (Ct), the cause of trachoma, may be useful for surveillance following population-level interventions, which include antibiotic mass drug administration (MDA). More information may be generated by analysing antibody levels rather than simply estimating seroprevalence.

Methodology

We used the level of antibodies against Pgp3, a Ct-specific antigen, to examine the intensity of Ct transmission at population level, using R package 'tmleAb'. Dried blood spot or serum samples from seven countries where trachoma is either currently or was previously endemic were employed. Levels of IgG against Pgp3 were measured using either a multiplex bead assay (MBA) or an ELISA. Semi-quantitative antibody levels were used to create age-dependent geometric mean antibody level curves.

The following comparisons were made: 1) pre- and post-MDA antibody levels from individuals of all ages in Nepal were compared with post-MDA data from Tanzania; 2) antibody levels from individuals of all ages in two regions of The Gambia, one of which had previously received MDA; 3) antibody levels in children aged 1–9 years from peri-elimination populations in The Gambia, Uganda, and Laos were compared with those from children in Kiribati, a country with ongoing transmission of ocular Ct; and 4) antibody levels from individuals of all ages in The Gambia were compared with those from residents of the Solomon Islands, a country with a high prevalence of urogenital Ct infection.

Significance

In children from populations in which trachoma has been, or is close to being, eliminated as a public health problem, there is little or no increase in mean antibody levels with increasing age. In populations with a high prevalence of urogenital Ct infection, there is a rapid increase in signal at the age of sexual debut. Wide confidence intervals around age-standardised means suggest increased exposure to antibody-inducing Ct infections.

6.2 Introduction

Ocular infection with *Chlamydia trachomatis* (Ct) causes trachoma, the leading infectious cause of blindness [1]. Trachoma is targeted for global elimination as a public health problem by 2020 [2]. Elimination requires the district-level prevalence of the active trachoma sign trachomatous inflammation—follicular (TF) to be <5% in 1–9-year-olds, and the district-level prevalence of trachomatous trichiasis (TT) unknown to the health system to be <0.2% in ≥15-year olds [3]. Any district in which the prevalence of TF and/or TT remains above the elimination threshold is targeted for some or all of the four components of the “SAFE” strategy: Surgery for TT, Antibiotics to clear infection, Facial cleanliness and Environmental improvement, particularly better access to water and sanitation. The A, F and E components of SAFE are intended to reduce the transmission of ocular Ct infection [1].

Recent evidence has shown that high prevalence of TF in 1–9-year-olds does not always correlate with high prevalence of ocular Ct infection [4], that TF can occur in the absence of any bacterial infection [5] and that a follicular conjunctivitis resembling TF may be common among people who have never had an ocular infection with Ct [6]. In light of this there are some concerns that antibiotic mass drug administration (MDA) might be administered in populations where there is no (or very limited) ongoing transmission of ocular Ct. There have been calls for the use of assessment methods that more directly measure the extent to which Ct is prevalent within a community [7,8]. Serological tests that could be used to measure the prevalence of antibodies against Ct have been proposed as alternative or additional measures for post-MDA surveillance surveys [9], which the World Health Organization (WHO) recommends should take place at least two and a half years after the last round of MDA [10].

Pgp3 is a highly specific and immunogenic Ct antigen [11,12] and has been used extensively in serological studies of both ocular [9,13–24] and genital Ct infections [12,25–36]. Previous studies have evaluated the use of the age-specific prevalence of serum antibodies against Ct (classifying individuals either as seropositive or seronegative) as a potential marker of the intensity of transmission of ocular Ct infection [8,19,21,22,24]. Additional studies have used the prevalence of antibodies against Ct to estimate the seroconversion and seroreversion rates following ocular Ct infection [17,18]. Studies to date have measured seroprevalence by using thresholds to dichotomise semi-quantitative antibody measurements as positive/negative results, but resulting prevalence estimates can vary widely depending on the reference population and method used [17,37].

Other approaches to serological analysis have proved useful elsewhere. For instance, the use of antibody acquisition models has been of benefit in malaria studies in populations with declining transmission. These models produce more precise estimates of transmission patterns than seroprevalence and may better estimate transmission intensity, particularly in low prevalence settings [38]. Ensemble machine learning has

meanwhile been used to fit age-dependent antibody curves for diverse pathogens that may be included in multiplex bead assays (MBA) [39]. Such analytical methods use semi-quantitative antibody results, such as those from ELISA or MBA, and remove the need to define a threshold between seropositive and seronegative samples. This may provide more detailed and, crucially, more consistent findings between studies, especially in settings with very low transmission intensity which would otherwise require a very large sample size [40]. Semi-quantitative data are also of intrinsic value where transmission is in decline, as in such an environment the average age of first infection is likely to increase, and the frequency of repeat infections to diminish. Both of these factors would contribute to a slower increase in population-level antibody levels with age [39]. Antibody acquisition models therefore have the potential to reflect transmission more appropriately than do analyses using proportions of individuals who demonstrate responses above investigator-defined thresholds.

We used a new ensemble machine learning approach to antibody acquisition modelling to compare age-dependent antibody curves from a series of cross-sectional population-based trachoma studies. This novel analysis using R package 'Targeted maximum likelihood estimation for antibody measurements' (tmleAb) [39] includes an ensemble machine learning approach to antibody acquisition modelling which is computationally advanced but straightforward to implement with the open-source statistical analysis software R [41]. The populations studied include pre-, peri-, and post-elimination communities, with antibody levels measured using both ELISA and MBA. Age-specific seroprevalence data from these studies, using various methods to determine the cut-off between seronegative and seropositive, have been previously published [17,18,20,21,23,42]. We hypothesise that the age-antibody level curve in populations in which trachoma has been eliminated as a public health problem will have a characteristic profile, differing significantly from that derived from populations in which Ct infection remains a problem.

6.3 Methods

6.3.1 Ethics

This study was a secondary analysis of de-identified data. All of the antecedent studies [17,18,20,21,23,42] from which data were sourced had received approval from the relevant ethics committees or review boards. Ethical approval for secondary analysis of the data was received from London School of Hygiene & Tropical Medicine (Ref 16187). All studies were conducted in accordance with the Declaration of Helsinki.

6.3.2 Datasets

Table 6.1 summarizes the datasets used in the antibody acquisition analysis, including the population demographics, assay used, seroprevalence, and disease and infection prevalence for each study.

6.3.3 Measuring effects of MDA on antibody levels

Serological data were compared between pre- and post-MDA populations in 4 villages in Kapilvastu District of southwest Nepal [23]. In 2000–2002, serum samples were collected from participants of all ages and stored at -20°C. The prevalence of TF in children aged 1–9 years was 17.6% (95% Confidence Interval: 9.4–29.7). Three annual rounds of MDA were administered (2007–2009) and in 2014, blood samples were collected from the population. For children under 15 years of age, dried blood spots (DBS) were collected while serum samples were collected from participants aged 15 years and older. Disease prevalence was measured again in 2014: the prevalence of TF had declined to 0% (95CI: 0-5.6) in 1–9-year olds (who had not yet been born at the time of the previous survey) [23]. Serum samples from 2000–2002 and serum and DBS from 2014 were analysed. The prevalence of antibodies against Pgp3 was 59.6% (95CI: 46.1–71.8) in the first sample collection: the prevalence of antibodies was 3% in the samples collected in 2014 [23].

Antibody levels from Nepal were compared to those from a Tanzanian village in which ocular Ct infection was eliminated in 2005, following two rounds of high-coverage (>90%) MDA [18]. This study saw the prevalence of ocular Ct infection decline from 9.5% (95CI: 7.8-11.6) prior to the first round of MDA in 2000 [43] to 0% (95CI: 0-0.01) in 2005, three years after the second round of MDA [44].

6.3.4 Evaluating antibody levels in a peri-elimination setting

In The Gambia, despite a secular decline in trachoma prevalence from 1986–2006 associated with improved access to water and sanitation [45–50], the TF prevalence remained above the elimination threshold in some areas. Antibiotic MDA was therefore required in several regions of the country in 2007–2010 [46,48,51], including Lower River Region (LRR) but not Upper River Region (URR). The Gambia was subsequently considered to be in the peri-elimination phase. The most recent study, which examined samples of residents of all ages across LRR and URR in 2014, showed low prevalence of TF (3.4%), and a seroprevalence across the study population ranging from 4% in 1–9-year-olds to 63% in those aged 40 years and older [20,52].

Antibody levels were compared between the two regions.

6.3.5 Comparing Ct exposure in children

DBS were collected from children aged 1–9 years in Laos, Uganda and Kiribati [17,21]. In Laos, samples were collected from in three provinces considered to be potential trachoma “hot spots” [53]. Specific trachoma elimination interventions, including antibiotic MDA, have never been undertaken in Laos; the country was recently validated as having eliminated trachoma as a public health problem [54]. Samples from Uganda were collected as part of an impact survey, following three years (2010–2012) of implementation of the A, F

and E components of the SAFE strategy. DBS were collected from children in Kiribati in work coupled to surveys supported by the Global Trachoma Mapping Project [55], which was designed to determine the need for interventions against trachoma. Antibody levels from children in these three countries were compared to those from children aged 1–9 years in The Gambia, as described above.

6.3.6 Visualising the relative impact of urogenital and ocular Ct infections on antibody levels

DBS were collected from participants of all ages in the Solomon Islands, where there is a low prevalence of ocular Ct infection (1% in children aged 1–9 years) [4] but high prevalence of urogenital Ct infection [56,57]. The prevalence of urogenital Ct infection has been estimated to be as high as 28% in women under the age of 30 years in Honiara, the capital city [56], although this may have declined following azithromycin MDA given as part of trachoma elimination efforts [57]. Antibody levels were compared to those from individuals of all ages in The Gambia, where there is a low prevalence of ocular [48] and genital Ct infection [58,59].

6.3.7 Specimen handling

Sample management has previously been described for each dataset, but in brief: specimens from The Gambia, Laos, Uganda, Solomon Islands and Kiribati were shipped to the London School of Hygiene & Tropical Medicine, London, UK, for testing. They were stored at -20°C before being assayed using a Pgp3-specific ELISA [17]. Samples from Nepal and Tanzania were shipped to the US Centers for Disease Control and Prevention, Atlanta, USA, at ambient temperature (for DBS) or on cold packs (for sera), and stored at -20°C before being assayed for antibodies against Pgp3 using MBA [24].

6.3.8 Serological analysis of anti-Ct-antibodies

Specimens from The Gambia, Laos, Uganda, Solomon Islands and Kiribati were assayed using a Pgp3-specific ELISA as previously described [17]. In brief, serum was eluted from DBS then applied to a plate coated with Pgp3 protein; known standards were included on each plate and assayed in triplicate. Following incubation, bound antibody was detected using HRP-labelled mouse anti-human IgG(Fc) (Southern Biotech, Birmingham, USA). Plates were incubated and washed and then developed using 3,3',5,5'-tetramethylbenzidine (TMB, KPL, Gaithersburg, USA). The reaction was stopped with 1 N H₂SO₄ and optical density was read at 450 nm (OD₄₅₀) on a Spectramax M3 plate reader (Molecular Devices, Wokingham UK). Readings were corrected for background by subtracting the average absorbance of three blank wells containing no serum using Softmax Pro5 software (Molecular Devices, Wokingham, UK).

Specimens from Tanzania and Nepal were assayed using an MBA as previously described [24]. Briefly, Pgp3-coupled beads were added to each well of a filter plate (Millipore) and washed. Control sera and serum eluted from DBS were added in duplicate then incubated. Following incubation, antibodies were detected with biotinylated mouse anti-human total IgG (Southern Biotech, Birmingham, AL) and biotinylated mouse antihuman IgG4 (Invitrogen, South San Francisco, CA). Plates were incubated and washed then developed with R-phycoerythrin-labelled streptavidin and a solution of bovine serum albumin, Tween 20 and sodium azide in PBS. Beads were suspended in PBS, shaken, and immediately read on a BioPlex 200 instrument (Bio-Rad, Hercules, CA) equipped with Bio-Plex Manager 6.0 software (Bio-Rad). The median fluorescence intensity (MFI) for each sample was recorded and background from the blank well subtracted out (MFI-BG).

6.3.9 Statistical analysis

Statistical analyses were carried out using R [60]. Each cross-sectional survey captured age, gender, signs of trachoma, community of residence and other characteristics. These data were combined with laboratory results which in some instances included results from nucleic acid amplification tests for ocular Ct infection (Table 6.1). A nominal value was added to zero data: 0.001 for ELISA data, 1 for MBA data.

The R package 'tmleAb' [39] was used to fit age-dependent antibody curves. This package uses data adaptive ensemble learning [61] based on multiple models to create an algorithm which is used to minimise the variance in the distribution of data. In this case, the algorithm was based on the 'SuperLearner' ensemble [61], and included a generalised additive model, generalised linear model, geometric mean, local regression (loess) and Yman [38], for targeted maximum likelihood estimation [62] of antibodies by age group [63].

Because the range of possible values varied between ELISA (which measures absorption in units of optical density) and MBA (which measures fluorescent intensity in units of median fluorescent intensity [MFI-background]) data, the tmleAb script was modified to exponentiate the ELISA data, adjusting for the reduced range of possible values. Also for this reason, datasets generated using the MBA platform (Nepal and Tanzania) could not be compared to those generated using ELISA (The Gambia, Uganda, Laos, Kiribati and the Solomon Islands).

Data between studies were compared using age ranges that were common between studies. The parameter of interest was the marginal difference between age groups in geometric mean antibody levels. P-values for the geometric mean antibody level by age group were calculated accounting for stratified data (within age bands) and adjusted using a Bonferroni correction [39].

6.3.10 Seroprevalence estimation

Seroprevalence data obtained in each of the data sets were previously published [17,18,20,21,23,42].

Thresholds used to establish seropositivity were determined either by using finite mixture modelling [17] or by receiver operator characteristics (ROC) curves [24].

6.4 Results

6.4.1 Effect of MDA on antibody levels

In Nepal, pre-MDA data show a sharp increase in antibody levels with age in the youngest age groups, reaching a plateau around age 10 years then remaining high in older age groups. In contrast, there was a much slower rise in levels of antibodies in younger participants post-MDA (Figure 6.1A). The post-MDA antibody curves did not reach a plateau until around age 50 years. Until age ~20 years there was at least a \log_{10} difference in geometric mean antibody levels between age-equivalent participants in the two surveys: for example the average 10-year-old in post-MDA Nepal had 100 times lower antibody levels against Ct than the average 10-year-old in the pre-MDA population. The antibody acquisition model shows lower levels of antibodies in the youngest participants, with a sustained and significant difference between the pre- and post-MDA populations. The confidence intervals (CI) surrounding the mean estimates (Figure 6.1B) provide additional information, suggesting that not only were average antibody levels higher in children in 2002, but also that the absolute majority of children aged 6–10 years in 2002 had high levels of antibodies whilst the majority in 2014 had negligible levels. There are wide CI associated with the geometric mean antibody level among 1–5-year-olds in pre-MDA Nepal; CI are narrower for other age groups in the pre-MDA population and for all ages they are wider than the CI for the corresponding age group in post-MDA Nepal. In 2014 there was no period in which the geometric mean antibody level varied to this extent, suggesting that no age group was (compared to others) at particularly increased risk of seroconverting (Figure 6.1B).

Figure 6.2 compares Nepal's pre- and post-MDA data to data from a community in Tanzania where transmission of ocular Ct was interrupted with MDA. The post-MDA data from Nepal follow the same curve as the data from Tanzania (Figure 6.2A), providing some empirical indication that the 2014 Nepal survey was conducted in a district in which ocular Ct transmission was low or negligible. The confidence intervals are wide amongst 21–30-year-olds in both Tanzania and post-MDA Nepal (Figure 6.2B), while in pre-MDA Nepal, confidence intervals are widest in the youngest age group.

Table 6.1. Datasets used in the antibody acquisition analysis

Samples analysed using Pgp3-specific ELISA								
Country	Survey pre- or post-MDA	Age range (years)	N	Antigen	Threshold (OD ₄₅₀)	Seroprevalence % (95CI)	TF prevalence in 1–9-year-olds % (95CI)	Infection prevalence % (95CI)
The Gambia [17,20]	post	1–90	1868	Pgp3	0.672	26.2 (24.3-28.3)	NA	0.5 (0-0.9) [48]
		1–9	738	Pgp3	0.672	6.4 (4.8-8.4)	1.6 (0.9-2.8)	
Uganda [17]	post	1–9	2700	Pgp3	0.5537	17.1 (15.7-18.6)	3.4 (2.8-4.8)	0.3 (0.1-0.6) [9]
Laos [17]	post	1–9	952	Pgp3	0.696	15.6 (13.5-18.1)	1.6 (1.0-2.6)	Not measured
Kiribati [21]	pre	1–9	397	Pgp3	0.245	53.0 (48.0-57.8)	28.0 (23.8-32.6)	24.0 (20.0-28.4)
Solomon Islands [4]	post	1–90	1511	Pgp3	0.7997	42.2 (39.8-44.7)	NA	Not measured
		1–9	458	Pgp3	0.7997	18.0 (14.7-21.7)	14.2 (11.3-17.7)	9.8 (4.6-19.9)*
Samples analysed using Pgp3-specific MBA assay								
Country	Survey pre- or post-MDA	Age range (years)	N	Antigen	Threshold (MFI-Bkgd)	Seroprevalence % (95CI)	TF prevalence in 1–9-year-olds % (95CI)	Infection prevalence % (95CI)
Nepal [23]	pre	2–80	659	Pgp3	801	77.1 (73.7-80.1)	17.6 (14.9-20.7)	Not measured
	post	3–90	646	Pgp3	801	34.9 (31.3-38.6)	0 (0-0.6)	Not measured
Tanzania [18]	post	1–87	557	Pgp3	1024	31.1 (27.4-35.0)	6.5 (4.7-8.8)	0 (0-0.7) [44]
<p>MDA=mass drug administration; TF=trachomatous inflammation—follicular; 95CI= 95% Confidence Interval; NA=not applicable. MFI-Bkgd=Median fluorescence intensity with background subtracted. OD450 = optical density at 450 nm. ELISA = enzyme-linked immunosorbent assay. MBA = multiplex bead assay.</p> <p>* Swabs for infection testing were only collected from the 61 children in whom TF was present.</p>								

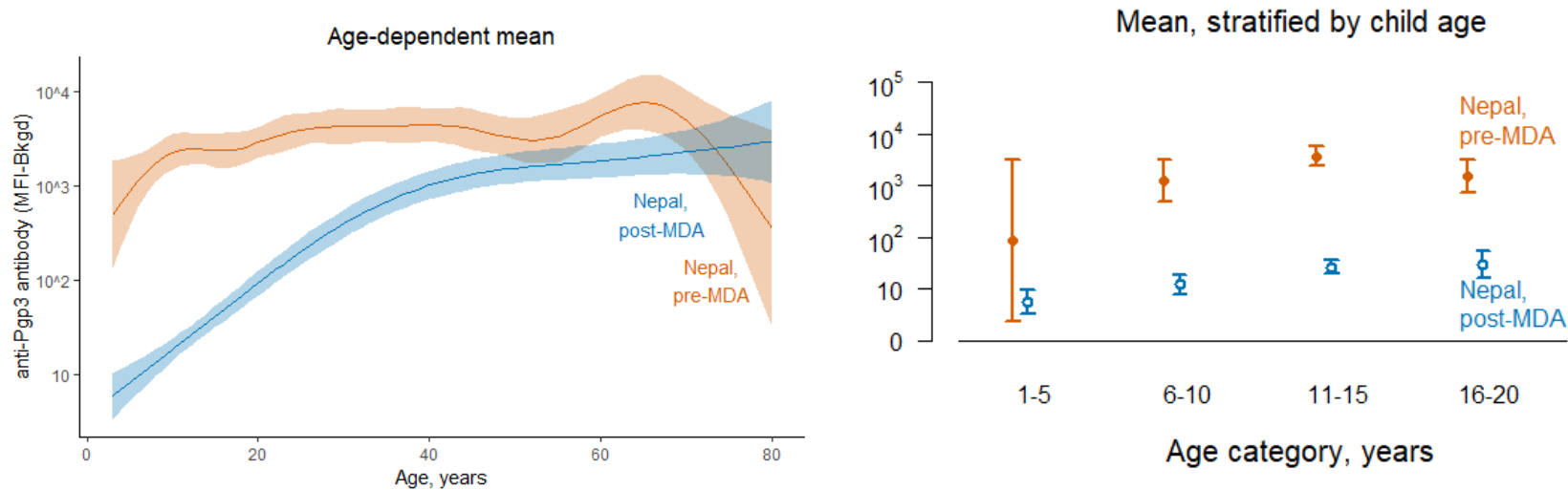


Figure 6.1. A shift in the *C. trachomatis* Pgp3 age-antibody level curves demonstrates a reduction in transmission following antibiotic mass drug administration (MDA) in Kapilvastu District, Nepal. IgG antibody responses to Pgp3 measured in serum sampled both prior to mass drug administration (MDA) in 2002 (n=659, orange) and after MDA in 2014 (n=646, blue). MDA was performed in three annual rounds (2007, 2008 and 2009). **1A** shows age-specific geometric means are shown as curves. Shaded bands show 95% confidence intervals. **1B** shows age-adjusted geometric mean antibody response (ages 1-20) and 95% confidence intervals for the same both before (2002, orange) and after (2014, blue) azithromycin MDA. Age is limited to individuals 20 years and younger to demonstrate the width of the confidence intervals in the youngest age group. Differences between means are significant (Bonferroni corrected $p < 2e-16$) except for the 1–5-year-olds age group. Data are stratified into 5-year age categories.

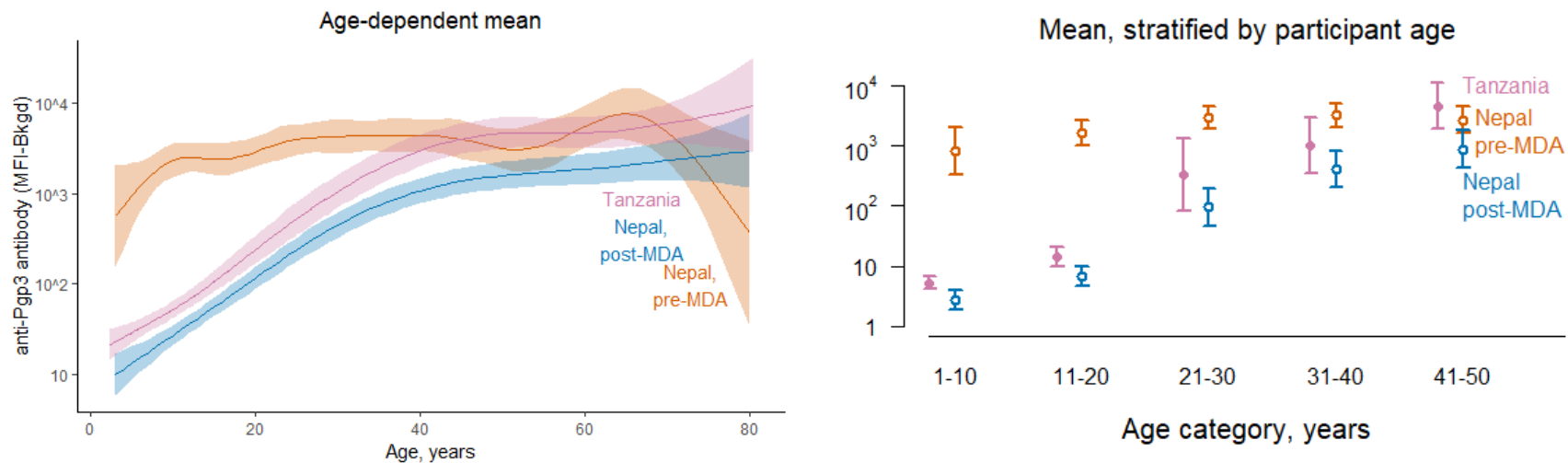


Figure 6.2. *C. trachomatis* Pgp3 age-antibody level curves from Kapilvastu District, Nepal, compared to a population in Rombo, Tanzania, where ocular Ct infection was eliminated in 2005 [44]. **2A** shows the geometric mean antibody levels by age and survey. Data from the pre-mass drug administration (MDA) Nepal population (2002, orange) is compared to the post-MDA Nepal population (blue, 2014) as well as a population from Rombo, Tanzania (pink) where ocular Ct infection was eliminated following antibiotic MDA. Shaded bands show 95% confidence intervals. **2B** shows age-adjusted geometric mean antibody responses in individuals aged 10–50 years and 95% confidence intervals for the two sets of samples from Nepal before (2002, orange) and after (2014, blue) azithromycin MDA and the post-MDA population in Rombo (pink). Differences between Nepal pre-MDA and Rombo means are significant (Bonferroni corrected $p < 0.002$) for the 1–10 and 11–20 years age categories, and (Bonferroni corrected $p < 0.2e-16$) for the 21–30 years age category. Data are stratified into 10-year age categories.

6.4.2 Antibody levels in the peri-elimination setting

Figure 6.3 shows the age-dependent geometric mean of anti-Pgp3 antibodies in the two Gambian regions studied: LRR and URR. Age-dependent means are equal between the two regions in the youngest participants and there is only a difference in the adult population (Figure 6.3A). Closer examination of the age-adjusted mean shows consistently low antibody levels across the youngest age groups, with narrow confidence intervals for the adjusted geometric mean antibody levels for participants aged 1–9 years; the width of the confidence intervals is slightly wider amongst 10–12-year-olds and 13–15-year-olds than in the younger age categories, while still overlapping with those of other age groups (Figure 6.3B).

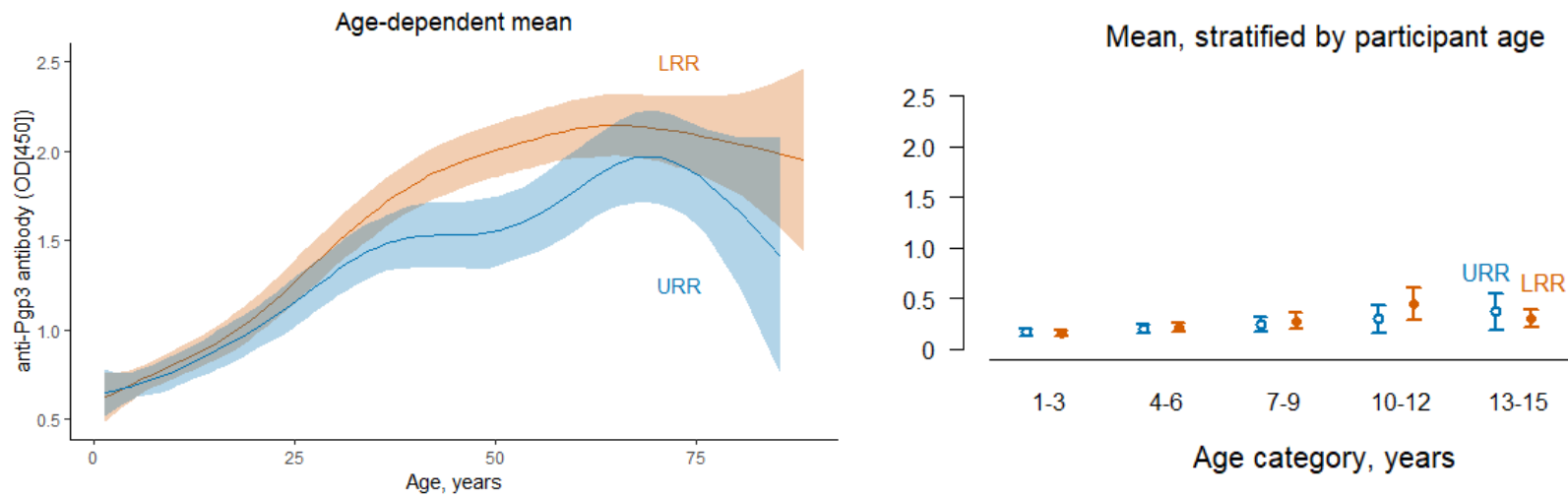


Figure 6.3. Variation in the *C. trachomatis* Pgp3 age-antibody level curves between populations in two regions of The Gambia. Communities in Lower River Region (LRR, N=1,010, orange) received three annual rounds of antibiotic mass drug administration (MDA), while those in Upper River Region (URR, n=822, blue) received none. **3A** shows the age-dependent geometric means. Shaded bands show 95% confidence intervals. **3B** shows the age-adjusted geometric mean antibody response for children aged 1–15 years with 95% confidence intervals for the two regions. Differences between means are not significant (Bonferroni corrected $p=0.619$). Data are stratified into 3-year age categories.

6.4.3 Comparing Ct exposure in children

Figure 6.4 shows the combined age-adjusted geometric means of children aged 1–9 years from four countries: Laos, Uganda, The Gambia and Kiribati. Disease and infection prevalence estimates in each setting are provided in Table 6.1.

The geometric mean anti-Pgp3 levels for Laos, Uganda and The Gambia are all similarly low: under 0.5 OD₄₅₀, and nearly horizontal. In contrast, the mean antibody level for Kiribati increases steeply with increasing age, and at all ages is higher than those for the other three countries, suggesting ongoing intense transmission of Ct. The confidence intervals are wider for every age group in Kiribati than in any other population included here.

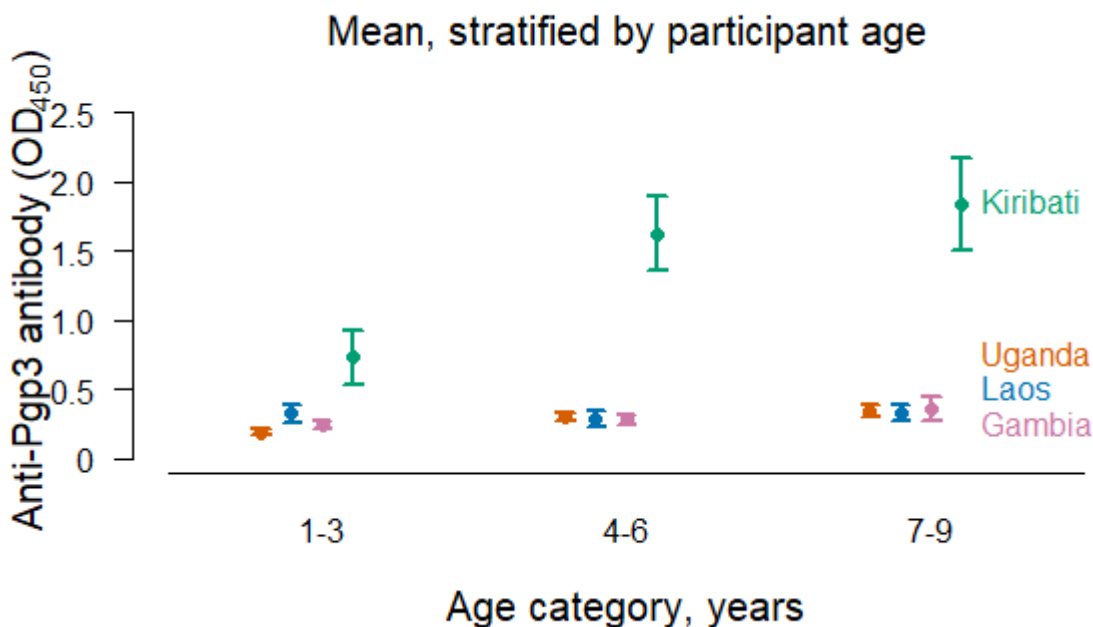


Figure 6.4. *C. trachomatis* Pgp3 age-antibody level curves between children aged 1–9 years in The Gambia, Uganda, Laos and Kiribati. The age-adjusted geometric mean antibody levels, including 95% confidence intervals, for Kiribati (green) compared to those from Laos (blue), Uganda (orange) and The Gambia (pink); data are stratified into 3-year age categories. The geometric means of age groups in Kiribati were compared to those from Laos, where trachoma has been certified as being eliminated. Differences between means are significant (Bonferroni corrected $p < 0.01$) for 1–3 years age group and (Bonferroni corrected $p > 0.002$) for 4–6 years age group and 7–9 years age group. 95% confidence intervals are shown. Data are stratified into 3-year age categories.

6.4.4 Relative impact of genital and ocular infections on antibody levels

Figure 6.5A shows the age-dependent geometric mean of anti-Pgp3 antibodies populations in The Gambia and the Solomon Islands across all ages. The summary curves from The Gambia shows a change in slope,

increasing around 20 years, while the summary curve for the Solomon Islands increases sharply from 15 to 30 years, then drops from 30 to 70 years of age before increasing again. For all ages, the summary curve in the Solomon Islands is higher than that of The Gambia. Figure 6.5B shows the geometric mean antibody level stratified in age groups with the geometric mean values from the Solomon Islands and The Gambia.

In both Figures 6.5A and 6.5B, the age-dependent mean antibody levels are higher in the Solomon Islands than in The Gambia. In the Solomon Islands the age-dependent antibody level begins to increase around 15 years, while in The Gambia, mean antibody levels increase at a lower rate after 20 years. Confidence intervals are wider in the Solomon Islands beginning in age group 21–30 and remain wide thereafter.

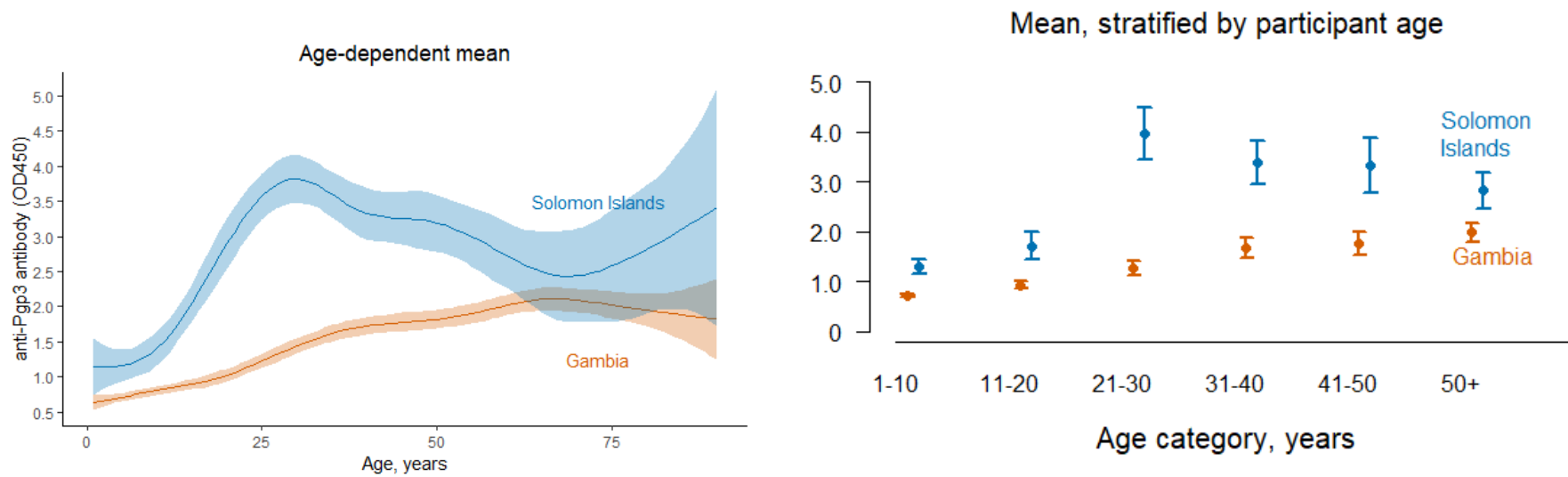


Figure 6.5. Variation in the *C. trachomatis* Pgp3 age-antibody level curves between The Gambia and the Solomon Islands. **5A** shows the age-dependent geometric mean in the Solomon Island samples (n= 1,499, orange) and The Gambia (n=1,805, blue). Shaded bands show 95% confidence intervals. **5B** shows the age-adjusted geometric mean antibody response by age category. Differences between means are significant (Bonferroni corrected $p < 0.01$) for 1-10 years and 50+ age groups and (Bonferroni corrected $p < 0.002$) for all other age groups, although optical densities measured at an intensity > 2.0 are associated with higher levels of error. Data are stratified into 10-year age categories.

6.5 Discussion

We have evaluated age-dependent antibody response to infection with Ct in seven different populations using an ensemble machine learning approach. Previous serological studies have assigned seropositive/seronegative status to samples, however determining thresholds for estimation of prevalence can be problematic [17]. This novel analysis, using R package 'tmleAb', removes the need to dichotomise continuous ELISA or MBA data and produces age-dependent mean antibody levels.

These age-dependent geometric mean antibody levels produce characteristic curves based on Ct transmission intensity at different ages. Samples collected from trachoma-endemic populations show a rapid increase in antibody levels with age in 1–9-year-olds, as seen in Kiribati (Figure 6.4), and continuing to rise steeply before plateauing around 50 years of age, as seen in the pre-MDA population in Nepal (Figure 6.1A and 6.2A). In post-MDA and peri-elimination communities, antibody levels increase much more slowly, as seen in the samples from post-MDA Nepal and Tanzania (Figure 6.2A) and The Gambia (Figure 6.3A). In children aged 1–9 years, the antibody acquisition curve is virtually horizontal in peri- and post-elimination populations (Figure 6.4), suggesting there is a characteristic shape to age-dependent mean antibody acquisition curves in populations where trachoma is no longer a public health concern. This could be used to support, or as an alternative indicator to, the clinical findings of TF. As decisions regarding implementation and cessation of MDA are based on the prevalence of TF in children aged 1–9 years, there may be no need to extend serological surveys to older age groups. Extending the survey to include adults may provide information about the prevalence of genital Ct infections, which can contribute to the prevalence of antibodies in the youngest participants through vertical transmission of Ct during birth.

Interestingly, the relative width of confidence intervals around age-stratified means may indicate when a population is under the greatest force of Ct infection, either ocular or genital. The confidence interval around the mean is impacted by the variability (as standard deviation) in the sampled population, the specified confidence level (95% in this analysis) and the sample size (all relatively similar in this analysis) [64]. Prior to infection with Ct, mean antibody levels are low, with narrow confidence intervals. When infection occurs within an age group, it is likely to occur asynchronously amongst different individuals in that group, thus producing a stimulus towards variability in the level of antibodies between individuals. In pre-MDA Nepal, when the prevalence of TF in 1–9 year olds was 17.6% (95CI 14.9-20.7), the widest confidence intervals were seen in the youngest population, children aged 1–5 years (Figures 6.1B and 6.2B). Similarly, in Kiribati, where TF prevalence in children aged 1–9 was 28.0% (95CI 23.8-32.6), confidence intervals are wide, particularly in comparison to children of the same age from Laos, Uganda and The Gambia, where TF prevalence was estimated to be 1.6% (95CI 1.0-2.6), 3.4% (95CI 2.8-4.2) and 1.6% (95CI 0.9-2.8), respectively (Figure 6.4), suggesting children in these population have only limited Ct infection. It would be useful to

examine age-dependent antibody level data from populations with higher estimations of seroprevalence to determine if our findings regarding wide confidence intervals hold true when seroprevalence rises greatly in a small age range, such as the population described by Goodhew and colleagues where 97% (95CI 93.9-98.7) of study participants were seropositive by the age of 6 years [15].

In populations with low levels of active trachoma in children, wide confidence intervals are seen in young adults around the age of sexual debut. The post-MDA populations in Nepal and Tanzania both had the widest confidence intervals in 21–30 year olds (Figures 6.1B and 6.2B). Confidence intervals were particularly wide for this age group in the Solomon Islands (Figure 6.5B), a country known to have a high prevalence of urogenital Ct infection [56,57]; The Gambia, in contrast, is thought to have a low prevalence of urogenital Ct infection [58,59].

This analysis has some limitations. The use of serology to monitor transmission of ocular Ct infection is not yet standardised. There are two assays commonly used for trachoma serological studies: a Pgp3-specific ELISA [17] and an MBA [24], which also detects antibodies against Pgp3. Data from the two assays are not directly comparable due to the distinct nature of each assay's output. Additionally, we were not able to compare all age ranges as some studies included only include children between 1–9 years of age as this is the age range of interest in measuring the prevalence of TF. This may be relatively unimportant as our analysis shows a distinct difference between communities with on-going ocular Ct infection and those with low levels, and this difference is obvious even in the limited age range of 1–9 years.

Using mean antibody levels removes the challenges of setting an appropriate threshold between seropositive and seronegative samples. By comparing antibody data from several studies, we have shown: a) a clearly significant separation between pre- and post-MDA populations; b) which ages of a population are under transmission pressure and acquiring Ct infections, either ocular or genital; c) the characteristic baseline and slope of a population in which elimination has been achieved; d) that it may be possible to segregate effects of ocular and genital Ct, at least in populations with low prevalence of ocular Ct infection and high levels of genital Ct infection.

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6.7 Author Contributions

Conceptualisation: SJM, ChR

Sample collection: SJM, PAM, RB, DD, OS, RT, AWS

Data Curation: SJM, DLM, AWS

Methodology: SJM

Supervision: ChR, DCWM

Writing original draft: SJM, ChR, DCWM

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Chapter 7- Discussion

Summary

In this final chapter, each research objective is summarised. Limitations to the work and to serology in general are discussed. Finally, practical considerations are presented, placing the work in this thesis in the greater context of NTD control and elimination, as well as future research possibilities which would apply to both genital and ocular infection with Ct

7.1 Summary of Research Findings

This section provides a brief overview of the findings of this thesis work in relation to the objectives set out in Chapter 1. For each objective, the research paper in which a detailed description of the findings is reported, is highlighted.

7.1.1 Objective 1

Collect demographic and clinical information and fingerprick blood samples to determine the prevalence of trachoma and antibodies against Pgp3 in Lower and Upper River Regions of The Gambia (Chapters 4 and 5)

A population-based prevalence survey was undertaken in Lower River Region (LRR) and Upper River Region (URR) The Gambia, using a two-stage sampling strategy with villages selected by probability proportional to size and households randomly selected a household list. LRR had previously undergone three rounds (2007-2009) of MDA of azithromycin for trachoma, while URR has never had specific trachoma elimination activities because trachoma has not been of a sufficiently high prevalence to justify intervention activities.

A total of 1,868 people of all ages participated in the study. The overall prevalence of TF was 1.6% (95CI 1.1-2.3%). Amongst 1-9 year olds, the prevalence of TF was 3.7% (95CI 2.2-6.1%) in LRR and 3.1% (95CI 1.7-5.4%) in URR [1], a non-significant difference ($X^2 = 0.1343$, $p = 0.714$) and in both regions the prevalence of TF in 1-9 year-olds was below the 5% threshold for elimination as a public health problem, as specified by the WHO [2]. Additionally, examiners found 78 cases of TS (71 cases in those >15 years), eight cases of TT and one case of CO (all in >15 years). There was a significant difference in TS prevalence between the URR (2.7%; 95CI 1.8-4.2%) and LRR (5.4%; 95CI 4.1-6.9%) ($X^2 = 7.2435$, $p = 0.007116$).

The prevalence of TF in children aged 1-9 years is below the 5% threshold for elimination as a public health problem [3].

Seroprevalence estimates suggest there is very little Ct circulating in the regions studied, as evidenced by the low seroprevalence in the youngest participants (Chapter 5) and corresponds to virtually non-existent levels of ocular Ct infection, demonstrated following the PRET study [4].

7.1.2 Objective 2

Develop a Pgp3-specific ELISA to measure antibodies against Ct using fingerprick dried blood spots (Chapters 3 and 4)

Previous work using serological assays to measure antibodies against Ct in trachoma-endemic populations was performed using a multiplex bead assay (MBA) and measured antibodies against Pgp3 and CT694 [5–7]. This assay was financially unviable for this thesis research thus with collaborators from the CDC, I optimised the protocol for a Pgp3-specific ELISA [1]. Serum standards were provided by the CDC and are described in detail in Chapter 3 and 4. Standards were tested in triplicate and standard deviations (SD) and coefficients of variations (CoV) were calculated based on the blanked OD₄₅₀ value.

For ongoing quality control, the mean OD₄₅₀ values, SD and CoV for the five serum standards were tracked across sample sets. Inter-plate variation was less than 15% across all plates in each set. This ELISA has been used by other researchers in surveys of the South Pacific, Malawi and Ghana [8–10] with similar results.

7.1.3 Objective 3

Determine appropriate thresholds to distinguish between seropositive and seronegative individuals in populations from The Gambia, Laos and Uganda (Chapter 4)

Both ELISA and MBA produce continuous numerical data as outputs which are typically converted into a simple binary positive/negative response. The thresholds used to determine positive/negative are based on reference standards. Mis-classification in the reference standards may introduce errors in the threshold between seropositive and seronegative samples. To overcome the need for reference standards, we explored alternative methods to establish thresholds, based only on the data generated during the study. These internally calibrated methods specified thresholds at approximately the same OD₄₅₀ value for each of the samples sets from the three countries in the study (Laos, Uganda and The Gambia). The finite mixture model (FMM) and expectation-maximisation algorithm (EM) are replicable and can allow for increasing sensitivity or specificity by changing the number of SD used by the algorithm to determine the threshold.

From a programmatic point of view, the absolute value and accuracy of the estimated seroprevalence is less relevant than the changes in population seroprevalence over time, particularly between pre- and post-MDA sampling of populations. Trachoma elimination programmes should consider collecting DBS as MDA is initiated. The fieldwork in this thesis has shown that DBS can be easily incorporated into trachoma surveys, following ocular examination.

7.1.4 Objective 4

Use seroprevalence data to estimate changes in transmission in The Gambia and estimate seroconversion and seroreversion rates (Chapter 5)

A large amount of effort and international funding has gone into the trachoma elimination efforts and it is important to monitor and evaluate the effectiveness of these efforts. Detecting a significantly reduced transmission of infection is a key indicator that elimination is likely to have been achieved.

Using the seroprevalence estimates from LRR and URR [1], estimates of seroconversion (SCR) and seroreversion (SRR) were used to explore the dynamics of Ct transmission in these two regions. The seroconversion and seroreversion rates were estimated under different epidemiological conditions using a reversible catalytic model [11]. Using catalytic models for trachoma research is not a new concept, as it has previously been used to model disease prevalence [12,13], and combining modelling with serological data can help determine the force of infection (Fol) by using SCR as a proxy [14].

Our model compared three possible situations: i) a constant SCR, ii) an abrupt change in SCR at some point prior to sample collection, or iii) a constant SCR followed by a log-linear decay in SCR. The model allowing for an abrupt change in SCR showed the best fit, suggesting a change in SCR in LRR approximately 23 years before sample collection and in URR approximately 16 years before sample collection. This interpretation may be confounded by considering an alternative scenario, wherein participants are exposed to a genital chlamydial infection at some more contemporaneous time.

Detecting a change in SCR additionally depends on both the sample size as well as the scale of change in SCR, with smaller changes in SCR requiring larger sample sizes.

7.1.5 Objective 5

Use antibody level data as an alternative to seroprevalence data to measure changes in Ct transmission (Chapter 6)

Antibody-level data was used to generate age-dependent antibody curves for comparison using a recently developed statistical package, 'tmleAb' [15]. I compared: countries pre- and post-intervention, regions with different scales of intervention activities, and countries with varying prevalence of ocular and genital Ct infections. My analysis showed that there is a characteristic shape to the curve in communities where trachoma is no longer a public health problem, with a

nearly horizontal curve in the youngest age groups, which increases slowly with age. In populations with low prevalence of ocular Ct infection but where genital Ct infection is prevalent, the slope increases rapidly around the age of sexual debut, then stays elevated. When comparing pre- and post-MDA populations, there is a statistically significant difference in the age-dependent mean antibody levels, shown by comparing pre-MDA data from Nepal to post-MDA data from the same population [16]. The post-MDA data was statistically similar to data from a post-MDA community in Tanzania [17,18]. Similarly, children (1-9 year olds) in Kiribati [19] had statistically higher mean antibody levels than their peers of the same age in Laos, Uganda and The Gambia, all regions that are post-MDA [1] or have been declared to have eliminated trachoma as a public health problem [20].

Confidence intervals around the age group-dependent means are widest in the age groups that are experiencing the force of Ct infection, whether ocular or genital. The 95% CI are wider in young children in trachoma-endemic regions- pre-MDA Nepal and Kiribati- while in regions in which trachoma is no longer considered a public health problem, wider confidence intervals are often present at the age when sexual debut occurs. This is most obvious in the Solomon Islands, where there is a high prevalence of genital Ct infection [21,22], but can also be seen in post-MDA populations in Nepal and the Solomon Islands.

Based on the above, age-dependent antibody levels are a practical means of monitoring Ct infections in a population, eliminating the need to set a threshold between seropositive and seronegative samples.

7.2 Limitations

There are limitations to the analyses presented in this thesis. Some are due to practical restrictions, while others require a reconsideration of trachoma elimination programmes

7.2.1 Study design

Most studies are cross-sectional, covering a single point in time; few studies collect serological samples at two or more time points [6,16,23]. An ideal study would collect samples from communities prior to the implementation of any interventions, then again during and following completion of the SAFE strategy. However, this thesis included one study with pre- and post-MDA samples [16], which showed a significant reduction in age-dependent antibody levels from pre- to post-MDA population and produced a similar age-dependent antibody profile to a population in Rombo, Tanzania where trachoma and ocular Ct infection were eliminated following MDA [17,18]. This thesis also compared three populations with low prevalence of both trachoma and ocular Ct

infection [1], two of which had previously undergone MDA with azithromycin, to a population with high prevalence of trachoma that had yet to undergo MDA [19]. The antibody levels in the pre-MDA population were significantly higher than those of the three low prevalence populations, which all had similar levels of antibodies.

7.2.2 Population to be sampled

There is no consensus on which age range should be surveyed for serological studies. Thresholds for the initiation or cessation of programmatic MDA are based on the prevalence of TF in children aged 1-9 years and many studies have focused on these children for serology as well [6,23,25–28]. In principle, the serological history of individuals born before the reduction in F_{ol} (older) consists of a time period at a high SCR which is then followed by a second period at lower SCR. Those born after the change in SCR (younger) will have experienced a constant SCR [29]. DBS were collected from participants of all ages in The Gambia; datasets from Nepal and Tanzania also comprised samples from participants of all ages. All modelling performed as part of this thesis was applied to data from either a wide age range (1-90 years) or a more limited age range focusing only on children. Increasing the age range of participants involved in a study to include adults may result in confounding if levels of genital Ct are high, however data from Chapter 6 suggests it may be possible, at population-level, to use confidence intervals to estimate when Ct infections are being acquired. Using age-based confidence intervals may allow for researchers to ascertain whether Ct-specific antibodies are being produced as a result of ocular or genital Ct infections.

7.2.3 Seroconversion

Secondly, most of the analyses assume persistent antibody levels and that both seroprevalence and antibody levels in participants have not declined significantly since they were initially infected with Ct. In Chapter 5, the SRR was estimated to be 0.008 and 0.010 in two Gambian populations. These calculations could be made more robust by examining a population at multiple time points following elimination, as has recently been demonstrated [24].

A study conducted in Tanzania showed no seroreversion in 1-6 year-old children six months following MDA [16], however a study in which children aged 1-9 years were followed up one year after baseline showed that seroreversion did occur, primarily in participants with antibody levels close to the threshold between seropositive and seronegative [23]. Following up a large population

of all ages may show that once antibodies are established above a certain level, for example following multiple ocular Ct infections in hyper-endemic populations, or multiple genital Ct infections, antibody levels remain fixed. This would support data in Chapter 6, where high antibody levels can be seen in the oldest population groups in Nepal, Tanzania, The Gambia and the Solomon Islands. The ability to detect a change using catalytic modelling assumes that an abrupt reduction in the Ct transmission intensity will translate into a similar change in SCR.

7.2.4 Assay Limitations

The Pgp3 antigen used in this ELISA was derived from serovar D, a urogenital strain of Ct. Another Pgp3-specific ELISA existed when this research was carried out [30] which was based on serovar L1, associated with LGV. Other Pgp3-specific ELISAs have been since been developed, one based on serovar E [31], as well a double antigen sandwich ELISA based on serovar L1 [32,33]; these other ELISAs have been used to study the prevalence of antibodies due to genital Ct infection. Only the double antigen ELISA has been compared to the original, indirect Pgp3 ELISA [32], however the other ELISAs, including the one used in this study, have not undergone head-to-head comparison.

Finally, there remains no gold standard references for antibodies resulting from Ct infection. Previous studies have used serum samples from children in non-endemic populations as negative controls, however these have shown to include samples that are considered seropositive based on their antibody levels. Similarly, positive samples have included infection-positive samples that would be classified as seronegative based on the antibody levels measured.

7.3 Practical Considerations

The research presented in this thesis can be considered translational in its nature, extending beyond the standard 'bench-to-bedside' [34] to 'bush-to-beside'. As such, application to practical public health outcomes is dependent on a variety of factors.

7.3.1 Sampling frame

Estimates of seroprevalence depend on the population sampled. As shown in Chapters 5 and 6, it is important to consider the age of people sampled and whether they are likely to have been exposed genital Ct infections through sexual activity, in addition to ocular Ct infection.

The size of the population to be sampled must be considered: the WHO recommends that trachoma (disease) prevalence estimates should be collected at the district level, with population sizes between 100,000 and 250,000, as this is the normal administrative unit for SAFE implementation [2]. It is likely district-level estimates of age-specific seroprevalence would also be sufficient, provided probability-proportional-to-size sampling rather than convenience sampling is followed. This also assumes in post-MDA districts, a low seroprevalence in young people is associated with a decline in the transmission of ocular Ct infection. The sample size required will depend on the population structure and *a priori* knowledge or estimation of disease and infection prevalence in the population to be sampled. Depending on the shape of the population pyramid within a country, it may be necessary to over-sample the youngest proportion of the population to achieve the power necessary to confidently measure changes in seroprevalence [35]. A second consideration is the time between the change in SCR and sample collection [29], such that there is a sufficiently large proportion of the population born after the change in SCR to compare to the older proportion. In areas with a high prevalence of genital Ct infection, oversampling around the age of sexual debut may elucidate additional patterns of infection [36]. Sampling frames should be guided by local knowledge of the epidemiology and prevalence of genital Ct infection.

7.3.2 Assays and data

Few studies to date have assayed samples in-country; the samples analysed as part of this thesis were assayed at either LSHTM or the CDC. With the exception of the lateral flow assay [28,37,38], assays to measure antibodies against Pgp3 are based on ELISA or MBA technology and require expensive reagents, many of which must be kept cold or frozen prior to use, and a continuous source of electricity. This may not be practical in trachoma-endemic countries and may require collaboration with a research or academic institute elsewhere.

Interpretation of ELISA or MBA data is straightforward when presented as seroprevalence data, can be easily understood and has been well-published using datasets from around the world. The interpretation of antibody level data is more complex and still a relatively new concept, not only for trachoma but for neglected tropical diseases in general [15]. This thesis contributes to the reservoir of knowledge about Ct serology and analysis and interpretation of antibody levels.

7.3.3 Following successful MDA

As more countries work towards the elimination of trachoma as a public health programme, MDA strategies may result in over-administration of antibiotics. This could potentially lead to antimicrobial resistance in Ct and commensal or opportunistic bacteria. A recent study has shown no antimicrobial resistance in *Mycoplasma genitalium* following one round of MDA [39], though there is concern that *Salmonella typhi* could easily develop resistance to azithromycin [40]. One possible solution to prevent over-administration is household targeted treatment, wherein households with one or more members presenting with active trachoma are treated, as is the current policy in The Gambia [36]. Because trachoma clusters by household [41–43], targeted treatment with a high level of coverage has been modelled to be cost-effective and to reduce the prevalence of ocular Ct infection [44].

MDA of azithromycin has been shown to reduce the prevalence of genital Ct infection [22] as well as reducing childhood mortality [45], and there may be other trade-offs to discontinuing MDA of azithromycin for trachoma elimination. This will need to be considered by policymakers in affected countries and NGOs involved with trachoma elimination programmers.

7.3.4 Symbiosis with other NTD programmes

The Expanded Special Project for the Elimination of Neglected Tropical Diseases (ESPEN) was established to accelerate elimination of NTDs for which preventive chemotherapy and transmission control (PCT) exist and to implement large-scale MDA. Along with trachoma, lymphatic filariasis, onchocerciasis, loiasis, schistosomiasis and soil-transmitted helminths are often co-endemic and therefore may benefit from collaborative efforts to accelerate treatment and prevention of these diseases [46]. As treatment for NTDs is expanded across all endemic regions, the incidence and prevalence of these disease will decrease, meaning case detection will become increasingly difficult and expensive [47–50]. Combining efforts for surveying, treatment and monitoring of intervention efforts across multiple endemic diseases may be one way in which to maximise resources. For example, the use of MBA to measure antibodies against a variety of NTDs would also allow for field surveys to collect information about a multiple diseases from a single DBS [51]. NTD programmes should consider combining efforts to maximise results, while reducing the demands of those affected by these diseases, donors and healthcare workers.

7.3.5 Future Research

Leading from this thesis, there are many areas for future research. A head-to-head comparison of ELISA and MBA results has not yet been published and doing so would allow for a direct comparison of the two techniques using the same research samples and standards. This would help inform assay choices in-country and the purpose for which each assay could be used. The development of serum reference standards would greatly facilitate this.

There is a need to further explore what level of antibodies may be associated with scarring sequelae, such that antibody level could be used to determine the risk of a person developing long-term sequelae. This would be relevant for both ocular and genital Ct infections, and in terms of intensified disease management, would help trachoma programmes follow up individuals at risk of progressing to trichiasis. For genital Ct infections, antibody levels could help determine the risk of infertility and the need for further medical intervention (I. Toskin, WHO, personal communication.)

Importantly, the research in this thesis was only able to compare one region for pre- and post-MDA antibody levels. With the increase in MDA for trachoma elimination that has occurred in the past decade [52] before the recent renaissance in Ct serology, it will not be possible to collect blood samples from pre-MDA populations. Serology should be incorporated in future MDA efforts to allow for monitoring changes in seroprevalence and antibody levels in communities. Combining efforts with other NTD programmes working in co-endemic regions could allow for on-going monitoring even once MDA for trachoma has been successful, and could be used to estimate more accurate seroreversion rates [24] once the FoI for trachoma reaches a non-significant level.

Finally, on-going studies have hypothesized that follicles on the upper tarsal may not solely be due to Ct, as seen in populations in the Solomon Islands [8]. Follicles may be due to a variety of non-chlamydial bacteria [53–55]; further consideration of the ocular microbiome and the role of commensal bacteria in good eye health should be explored.

7.4 Conclusions

The clinical results from my field work supports The Gambia's milestone of eliminating trachoma as a public health problem. Additionally, the analyses presented here support elimination in Laos and in the two regions studied in Uganda.

The data presented within this thesis substantiates the use of serology as an additional measure to monitor the prevalence of Ct infections and to support the decision-making process in trachoma elimination efforts. This is particularly demonstrated by the antibody prevalence data, which showed a consistent horizontal curve in children from post-MDA communities and regions where trachoma

has been eliminated as a public health problem. Ultimately, it may not be an exact seroprevalence or antibody level that is used as a metric for elimination, but the changes in seroprevalence or antibody levels over time.

In contrast to clinical indicators like follicles of the conjunctiva, which can be caused by other infectious agents, are not always correlated to Ct ocular infection and are subject to inter-grader variation, antibodies persist, can be (semi) quantitatively measured and those against Pgp3 are considered highly specific for infection with Ct.

A considered analysis of serological data may offer a complementary method to assess efforts to eliminate trachoma as a public health problem. Combined serological assays could gather information about multiple NTDs and infections, thereby maximising impact in resource-limited settings.

7.5 References

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Appendix 1- Eye exam and Dried Blood Spot (DBS) sample collection

Eye Exam and Dried Blood Spot (DBS) sample collection

Standard Operating Procedure (SOP)

V 19.11.2014

By Stephanie Migchelsen

Overview

This pilot project aims to measure the levels of anti-*C. trachomatis* antibodies in two populations: one where it is hoped that trachoma has been controlled through the recent mass administration of azithromycin and one where trachoma and the sequelae have been under control for many years.

Photographs will be taken of everted eyelids and blood samples, preserved on filter paper, will be collected from participants.

This project is designed to collect the following information from participants:

- Demographic information of participant
- Clinical phenotype according to the Simplified WHO trachoma grading system (Presence or absence of each of TF, TI, TS, TT, CO)
- Four dried blood spots from participants (at least 100 from each EA)
- Photographs of the conjunctiva (using predefined SLR photography protocol)

Written consent is required to take part in the follow-up project.

Blood spots must be dried before being stored in envelopes.

Staff and materials (per cluster)

Staff	Materials
2x MRC trachoma staff (grading and photography)	Information sheets
2 x Community Ophthalmic Nurses (CON) (blood and consent)	Inkpad for thumbprints
1 x field recorder	Pen(s) for signatures
1x driver	Consent forms
	Ocular Examination forms
	Census print-outs
	Binder to store forms
	Filter papers
	Lancets
	Alcohol swabs (EtOH+cotton)
	Storage bags
	Desiccant
	Sample storage box
	Drying sticks
	Printed labels
	Gloves
	Waste bag
	Nikon SLR camera
	Truck
	Fuel
	Table
	Chairs

Phase 1: Informed Consent

Step		Task
1 (Informed consent)	1.1	Read information sheet to household decision maker
	1.2	Invite decision maker to participate in study. Written consent required.
	1.3	If consent granted, a consent form must be signed for each member of the household who will take part in the survey.
		NOTE: Children below the age of 18 must have consent granted on their behalf by a parent or guardian. Children between 12 and 17 must provide their own assent.
		NOTE: Adults aged 18 or above must provide their own consent
	1.4	If consent is granted, proceed to step 2
2	2.1	Record ID number on Ocular Examination Form
	2.2	Ask demographic questions: Sex, Date of Birth/Age, and General Observations

Phase 2: Examination

2 (Examination)	2.1	Examine household head and any other adults who have consented to examination first
	2.2	Assistant calls ID to recorder for recording
	2.3	Grader uses 2.5x binocular loupes to examine eye in normal position.
	2.4	Grader examines for signs of TT and CO and calls verdict to recorder
	2.5	Grader everts right eyelid
	2.6	Grader examines for signs of TF and calls verdict to recorder
	2.7	Grader examines for signs of TI and calls verdict to recorder
	2.8	Grader examines for signs of TS and calls verdict to recorder
	2.9	Photographer takes photo of label
	2.10	Photographer takes photograph of everted right lid with SLR camera
	2.11	Photographer calls photo frame number to recorder

		NOTE: If lid eversion is too painful due to signs of trichiasis, the lid will not be everted and a photograph will be taken of the lid in normal position
	2.12	The right lid is returned to its normal position
	2.13	Steps 2.3-2.12 are repeated with the left eyelid
	2.14	Nurse collects one filter paper disk and one alcohol swab (cotton and ethanol)
	2.15	Nurse wipes participant's fingertip with alcohol swab, then pierces skin with the lancet
	2.16	Wipe the first drop of blood from the finger using the alcohol wipe
	2.17	Gently squeezing the fingertip, and holding the filter wheel by the label, use the filter wheel to collect at least four drops of blood, one on each of four outer circles. Both sides of the paper must be saturated with blood.
	2.18	Pass the filter paper to the assistant, who then places the wheel on a drying stick in the sample collection box.
		NOTE: Approximately 6 filter wheels can be placed on one drying stick. One finger-width must be kept between each wheel to ensure proper drying.
	2.19	Grader/Nurse thanks the examinee for their participation
	2.20	Grader changes gloves
		NOTE: Nurse to change gloves when (s)he thinks the gloves may be contaminated

Phase 3: Between clusters (end of the day)

Step		Task
3 (Storage)	3.1	Before the end of each day, the filter wheels must be removed from the storage box and placed individually in resealable plastic bags with a small amount of desiccant
	3.2	Data from Ocular Forms must be entered into database; photographs must be uploaded to Eyescores
4 (Charging)	4.1	Recorder must return to electricity source to enter data
	4.2	Assistants must charge photographic equipment each night

Appendix 3- Participant Information Sheet (Child)



PARTICIPANT INFORMATION SHEET (CHILD)

Version 1.0 Date 21/10/2014

Study Title: Age-specific seroprevalence of anti-*Chlamydia trachomatis* antibodies and the historical prevalence of trachoma

SCC:		Protocol:	
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Sponsor: Wellcome Trust

What is informed consent?

You are invited to let your child take part in a research study. Before you decide you need to understand why the research study is being done and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that is not clear or you do not understand. You may also wish to consult your spouse, family members, friends or others before deciding to let your child take part in the study.

If you decide to allow your child to join the study, you will need to sign or put a thumbprint on a consent form saying you agree for your child to be in the study. If your child is between the ages of 12-17, we will ask for his/her assent as a signature or thumbprint.

Why is this study being done?

One reason many people go blind in The Gambia is because of trachoma. Trachoma is an eye disease that is easily passed from person to person. If you get the infection many times it can lead to scarring in your eyelid, which causes the eyelashes to rub on the eyeball. This is painful and can eventually lead to blindness. When a person is infected with trachoma, they will produce antibodies to help fight this infection. These antibodies can stay in a person's blood for a very long time.

The National Eye Health Programme has recorded that over time, the amount of trachoma in many communities has gone down. We think this is because there is better access to latrines and water, as well as community screening for eye health, and giving antibiotics for infection.

A few years ago, some communities in The Gambia were given antibiotics against trachoma. We would like to compare communities that did receive antibiotics with communities that did not receive antibiotics because they did not need them.

We would like to check your child's eyes for signs of trachoma and test his/her blood to see if he/she has antibodies against trachoma.

The results of the study will be made available to your community.

What does this study involve?

We will ask 100 people of all ages in your community to have their eyes examined. We will examine the eye by gently turning over the eyelid. We will also take a small sample of

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blood. This will be done using a new, clean lancet to prick the fingertip. We will collect a few drops of blood onto a piece of paper.

If we discover your child's eyes cannot be examined because of an eye disease, we will refer him/her to the nearest eye health unit for appropriate care.

In case the investigator discovers your child is sick and decides that he/she cannot participate in the study because of that, he/she will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and your child will have the normal medical care.

What will happen to the samples taken in this study?

The samples will be taken back to the MRC laboratory in Fajara for testing to see if there are antibodies against trachoma. We will also send some of the samples to a lab in England to verify the results.

What harm or discomfort can you expect in the study?

We will prick your child's finger with a sharp pin to collect the blood. This can cause some discomfort to your child but the discomfort is not expected to last more than a day.

What benefits can you expect in the study?

While there are no direct benefits to you or your child, the information we get from this study will help us determine if the trachoma has been successfully eliminated from your community. This will help researchers make decisions that will help other communities around the world in their efforts to eliminate trachoma.

Will you be compensated for your child's/ward's participation in the study?

You will not get paid for participation of your child in the study.

What happens if you refuse to participate in the study or change your mind later?

You are free to let your child participate or not in the study and you have the right to stop his/her participating at anytime without giving a reason. This will not affect the medical care that your child would normally receive.

In case you decide to withdraw your child's participation during the study, we will not work on your child's samples without your permission, but any information already generated from the samples will be kept. The study doctor may also ask for tests for your child's safety.

Should any new information become available during the study that may affect your child's participation, you will be informed as soon as possible.

What compensation will be available if your child is injured during the study?

We will be responsible to provide for treatment caused by procedures of the research study. If medical treatment is required as an emergency, please refer to your health centre or clinic and contact the field worker who gave his/her telephone number to you or contact

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Stephanie Migchelsen on [Phone number] or Mr Sarjo Kanyi on 9901716, 3011349, 6651344 or 7510996.

How will your child’s information be kept and who will be allowed to see it?

All information that is collected about your child in the course of the study will be kept strictly confidential. Your child’s personal information will only be available to the study team members and might be seen by some rightful persons from the Ethics Committee, Government authorities and sponsor.

Who should you contact if you have questions?

If you have any queries or concerns you can contact Stephanie Migchelsen on [Phone number] or Mr Sarjo Kanyi on 9901716, 3011349, 6651344 or 7510996 and you can always call the personal numbers of the study staff given to you.

Please feel free to ask any question you might have about the research study.

Who has reviewed this study?

This study has been reviewed and approved by a panel of scientists at the Medical Research Council and the Gambia Government/MRC Joint Ethics Committee, which consists of scientists and lay persons to protect your rights and wellbeing.

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Appendix 4- Participant Information Sheet (Adult)

PARTICIPANT INFORMATION SHEET (ADULT)

Version 1.0 Date 21/10/2014

Study Title: Age-specific seroprevalence of anti-*Chlamydia trachomatis* antibodies and the historical prevalence of trachoma

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Sponsor: Wellcome Trust

What is informed consent?

You are invited to take part in a research study. Participating in a research study is not the same as getting regular medical care. The purpose of regular medical care is to improve one's health. The purpose of a research study is to gather information. It is your choice to take part and you can stop any time.

Before you decide you need to understand all information about this study and what it will involve. Please take time to read the following information or get the information explained to you in your language. Listen carefully and feel free to ask if there is anything that you do not understand. Ask for it to be explained until you are satisfied. You may also wish to consult your spouse, family members or others before deciding to take part in the study.

If you decide to join the study, you will need to sign or thumbprint a consent form saying you agree to be in the study.

Why is this study being done?

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What does this study involve?

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If we discover your eyes cannot be examined because of an eye disease, we will refer you to the nearest eye health unit for appropriate care.

In case the investigator discovers you are sick and decides that you cannot participate in the study because of that, you will receive immediate care at the study site and then be referred to the appropriate health facility.

If the research study needs to be stopped, you will be informed and you will have your normal medical care.

What will happen to the samples taken in this study?

The samples will be taken back to the MRC laboratory in Fajara for testing to see if there are antibodies against trachoma. We will also send some of the samples to a lab in England to verify the results.

What harm or discomfort can you expect in the study?

We will prick your finger with a sharp pin to collect the blood. This can cause some discomfort but the discomfort is not expected to last more than a day.

What benefits can you expect in the study?

While there are no direct benefits to you, the information we get from this study will help us determine if the trachoma has been successfully eliminated from your community. This will help researchers make decisions that will help other communities around the world in their efforts to eliminate trachoma.

Will you be compensated for participating in the study?

You will not get paid for participation.

What happens if you refuse to participate in the study or change your mind later?

You are free to participate or not in the study and you have the right to stop participating at anytime without giving a reason. This will not affect the medical care that you would normally receive.

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If you are injured in the study what compensation will be available?

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