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Assessment of IgA responses to *Chlamydia trachomatis* antigens among children from trachoma - endemic communities

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2011

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An abstract of
A thesis submitted to the Faculty of the
Rollins School of Public Health of Emory University
in partial fulfillment of the requirements for the degree of
Master of Public Health
in Hubert Department of Public Health
2013

Abstract

Assessment of IgA responses to *Chlamydia trachomatis* antigens among children from trachoma - endemic communities
By Sheri Maria Morgan

Trachoma, caused by the bacteria, *Chlamydia trachomatis*, affects millions of individuals worldwide, and is a leading cause of visual impairment and blindness. Nucleic acid amplifications tests are being used to confirm infection, but tests are expensive and are not routinely available in trachoma – endemic areas. After the introduction of mass drug administration (MDA), trachoma infection diminishes, but clinical signs may continue for a long period of time after infection prevalence declines, requiring new tools to determine if MDA is still warranted, and to carry out surveillance when MDA has stopped. We have previously shown that IgG response against the trachoma antigens, pgp3 and CT694, were associated with ocular pathology and active infection; however, many children with IgG antibody lacked evidence of either pathology or infection, indicating past exposure. In this study, we analyzed IgA responses to pgp3 and CT694 to determine if these provided a better marker of recent infection than IgG responses. Bloodspots were analyzed from a cohort of Tanzanian children (n=155) using an antibody – based multiplex assay to determine associations between IgA antibody responses, infection status, and ocular pathology. A second set of bloodspots were analyzed from a cohort of Tanzanian children (n=173) using an antibody – based multiplex assay to determine the kinetics of IgA antibody responses over time after MDA. Ocular swabs were analyzed for presence of *C. trachomatis* infection using Amplicor®. Antibody responses to both antigens were associated with infection status as determined by PCR. Only pgp3 antibody responses were significant when observing normal and TF/TI ocular pathology (pgp3 $p = 0.0207$, CT694 $p = 0.4691$). For children without evidence of trachoma or infection, 23% tested IgA-positive to pgp3, and 6% tested IgA-positive to CT694. These results suggest that IgA responses are not specific indicators of active infection. The results of the kinetics of IgA responses show a significant decline in both antigens six months after treatment for most age groups. Further studies with larger sample sizes are required to verify the findings of the kinetics of IgA antibody responses.

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Acknowledgements

First, I would like to thank my thesis advisor, Deborah McFarland, and committee member, Patrick Lammie for their constant and incredibly positive support. I am incredibly grateful for Sheila West and her research team at Johns Hopkins University Wilmer Eye Institute for allowing me to experience trachoma in an endemic country. I also thank the staff of the Kongwa Trachoma Project, in Kongwa, Tanzania, as well as all the individuals that participated in these studies.

Finally, I would like to thank my parents, Gamil and Sanaa, and brothers, Michael and Joseph, for being my continued inspiration.

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Acronym List

CO: Corneal Opacity

DALYs: Disability Adjusted Life Years

GET2020: Global Elimination of Trachoma by 2020

HALYs: Handicap Adjusted Life Years

HRS: High Risk Signs

IgA: Immunoglobulin A

IgG: Immunoglobulin G

ITI: International Trachoma Initiative

MBA: Multiplex Bead Assay

MDA: Mass Drug Administration

MFI – BG: median fluorescence intensity minus background

NTD: Neglected Tropical Disease

PCR: Polymerase Chain Reaction

PRET: Partnership for the Rapid Elimination of Trachoma

SAFE: Surgery, Antibiotics, Facial Cleanliness, Environmental Improvements

TF: Trachomatous Inflammation – Follicular

TI: Trachomatous Inflammation – Intense

TT: Trachomatous Trichiasis

UIG-A: Ultimate Intervention Goals for antibiotics

UIG-S: Ultimate Intervention Goals for trichiasis surgery

WHO: World Health Organization

Chapter 1: Introduction

1.1 Introduction and Rationale

Trachoma, caused by the bacteria *Chlamydia trachomatis*, is an ocular, neglected tropical disease causing severe morbidity and disability across the globe. Trachoma causes an estimated 3.8 million cases of blindness and 5.3 cases of low vision [1,2] in Africa and Southeast Asia. The disease is transmitted by direct personal contact, and also can be spread by flies that have come into contact with an infected person. If left untreated, repeated trachoma infection may progress from active infection, defined as, trachomatous inflammation – follicular (TF) or trachomatous inflammation – intense (TI), to scarring of the inside of the eyelid. Trachomatous trichiasis (TT), distinguishable by the turned-in eyelashes, results in damage to the cornea due to scratching from the eyelashes, and can lead to irreversible blindness caused by corneal opacity (CO).

Elimination efforts encompass The World Health Organi1zation's (WHO) Global Elimination of Trachoma by 2020 (GET2020) program is based on the components of the S.A.F.E. strategy. **S**urgery is used for trichiasis patients in order to prevent blindness, **a**ntibiotics are used as treatment for patients with active infection (TF/TI), **f**acial cleanliness is promoted to prevent the spread of infection from person to person, and **e**nvironmental improvements are through sanitation and hygiene are implemented in order to interrupt transmission. Pfizer has donated over 225 million doses of Zithromax (azithromycin) through distribution by the International Trachoma Initiative (ITI) (www.iti.org).

1.2 Statement of the Problem

As elimination programs reduce trachoma prevalence over time in a given country or geographic, defining programmatic endpoints becomes a priority. Clinical signs of trachoma may persist for individuals clear of active infection [3]. It is also possible that similar ocular pathology may be present in low prevalence settings, as inflammation may be caused by non-

Chlamydial bacteria [4]. Tests for active infection by Polymerase Chain Reaction (PCR) are available, but are costly. Currently, the WHO endpoint for antibiotic use is a follicular trachoma (TF/TI) rate of less than 5% in children under 10 years of age. However, clinical exams can be difficult to standardize between graders [5,6,7]. Therefore, alternative approaches to monitor trachoma prevalence over time are necessary.

Currently, antibody based tools are being investigated to explore their potential contributions in defining endpoints for neglected tropical disease (NTD) surveillance. Investigation of their utility for policy and programmatic decisions has so far included NTDs such as lymphatic filariasis, onchocerciasis, as well as schistosomiasis [8,9,10]. Antibody tests are also more cost effective when compared to the currently used polymerase chain reaction (PCR) technique. Previous studies have explored antibody assays for Chlamydia infection [11], and investigation of antibody assays for monitoring trachoma programs has recently begun [2]. Previous studies have also explored the sensitivity and specificity of various serological tools, and their capacity for evaluating trachoma transmission, defining endpoints of trachoma infection, and monitoring trachoma infection after mass drug administration (MDA) has ceased. Studies have also shown that a large percentage of children have detectable antibody responses, but exhibit no clinical signs, indicating prior infection [2]. This shows that there is a need for serological tools for measuring incidence or recent transmission. This study explores the contribution of the IgA antibody responses in order to determine indicators for recent trachoma infection.

1.3 Purpose Statement

The main goals of this project are to:

1. Analyze bloodspot samples obtained from trachoma endemic regions using immunological assays.

2. Use results from quantitative analysis in order to determine indicators for active infection, the kinetics of antibody responses, and cut offs for infection positivity.
3. Explore possible serological tools for trachoma programmatic use in the field to accurately determine infection, and monitor trachoma after MDA has ceased.

1.4 Research Hypothesis

The research hypothesis for this study is:

1. IgA serves as a marker for more recent trachoma infection due to more rapid clearance of IgA compared to IgG.
2. The threshold for infection positivity when detecting for IgA will be lower than that seen in IgG analysis due to lower IgA levels in human blood.

1.5 Significance Statement

Determining more robust endpoints for trachoma programs is a priority for elimination since the clinical signs may persist in the absence of active infection. As stated previously, PCR tests are costly, and antibody tests may serve as a more cost effective alternative to monitoring trachoma prevalence after mass drug administration (MDA) has ceased. Some antibody tests, such as the multiplex bead assay, have the capacity to observe a number of diseases in one analysis, and can also measure antibody responses for a number of individuals in a single assay run. Therefore, large scale surveys can be preformed for large populations and can also integrate a number of NTDs for mass prevalence screening. Exploring IgA as a potential marker for recent infection will help determine the incidence of trachoma infection in order to steer elimination programs. Since IgA responses are elevated during a first infection, and decrease rapidly over time after the first infection, increased IgA antibody responses may serve as an indicator of recent infection in a population. Determining recent infections in a population can help

differentiate past and recent infections, and will give public health practitioners and researchers a more accurate picture of trachoma transmission, as well as the progress and effectiveness of MDA efforts. Also, after MDA has ceased, immunological assays can serve as a cost effective measurement of trachoma infection. Negative IgA results would indicate that there are no recent infections of trachoma within the population, and the community has reached elimination.

1.6 Definition of Terms

Active Trachoma: a clinical exam of trachomatous inflammation – follicular (TF) or trachomatous inflammation – intense (TI)

Trachomatous inflammation – follicular (TF): the presence of five or more follicles in the upper tarsal conjunctiva. See Appendix 1 for image.

Trachomatous inflammation – intense (TI): pronounced inflammatory thickening of the upper tarsal conjunctiva that obscures more than half of the normal deep tarsal vessels. See Appendix 1 for image.

Trachomatous scarring (TS): the presence of scarring in the tarsal conjunctiva. See Appendix 1 for image.

Trachomatous trichiasis (TT): at least one eyelash rubs on the eyeball. See Appendix 1 for image.

Corneal opacity (CO): easily visible corneal opacity over the pupil. See Appendix 1 for image.

Low vision: corrected visual acuity in the better eye less than 6/18 but better than or equal to 3/60

Blindness: corrected visual acuity in the better eye less than 3/60

Health: the state of complete physical, mental and social well-being and not merely the absence of disease of infirmity

Visual loss: refers to the combined categories of blindness and vision loss

High risk signs (HRS): signs indicating that the trachoma infection prevalence is greater than 20% as defined by West and colleagues

Chapter 2: Literature Review

2.1 Introduction

The World Health Organization (WHO) recognizes health, defined as a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity, as a basic principle for the security of all peoples. WHO also recognizes health as a fundamental right for all human beings without distinction of race, religion, political belief, economic, or social condition [12]. Health intervention programs exist on multiple platforms and levels of organization, from the community level to a global scale. At-risk populations typically are the center of intervention programs in an effort to alleviate not only disease, but also illness. The WHO definition of health encompasses morbidity as well as mortality, as disability due to disease outcomes causes great social and economic burdens among at-risk populations. Neglected Tropical Diseases (NTDs) are known for their high morbidity and disability to their respective at-risk populations. The NTDs are 17 different diseases identified by WHO, and are listed in Table 1. NTDs cause substantial illness and stigma to the world's poorest individuals, and trap the poor within a continuous cycle of disease and poverty.

Control or elimination programs exist for each respective NTD, each with respective year goals. The goal for trachoma elimination is the year 2020, and is being executed by the implementation of the S.A.F.E. Strategy.

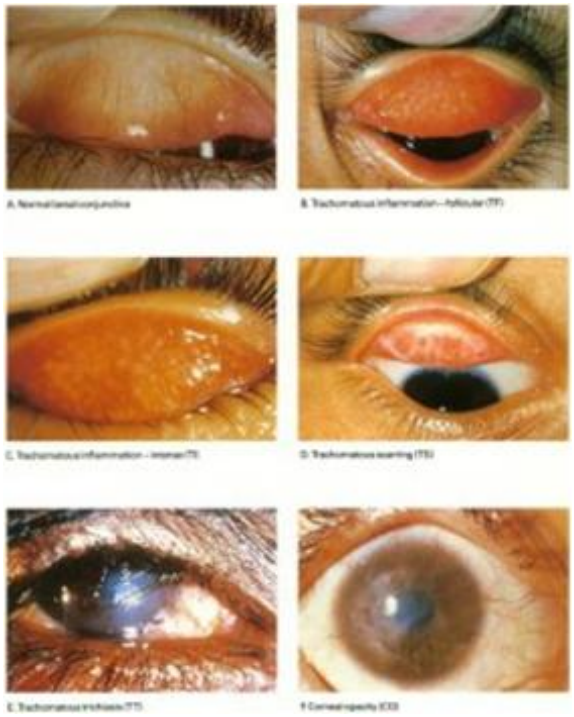
Table 1. The current list of Neglected Tropical Diseases as indicated by the World Health Organization Credit: WHO.

The Neglected Tropical Diseases (NTDs)	
Buruli Ulcer	Leprosy
Chagas disease	Lymphatic filariasis
Cysticercosis / Taeniasis	Onchocerciasis (River Blindness)
Dengue / Severe dengue	Rabies
Dracunculiasis (guinea worm)	Schistosomiasis
Echinococcosis	Soil transmitted helminthiases
Food borne trematode infections	Trachoma
Human African trypanosomiasis	Yaws
Leishmaniasis	

2.2 Trachoma Worldwide

Trachoma, caused by the bacterial agent *Chlamydia trachomatis*, is the leading infectious cause of blindness worldwide. Clinical trachoma manifestations are shown in Figure 1. Trachoma infection is highly correlated with low access to water and sanitation, poverty, and limited access to healthcare services [1,13]. Infection is spread by contact between person, and by the eye seeking female bazaar fly, *Musca sorbens* [14]. The flies serve as mechanical vectors of the disease by picking up pathogens from infectious material, such as dirt and pus around the eye of an infected child, and transferring them to an uninfected host by using their proboscis and legs.

Figure 1. The clinical manifestations of ocular trachoma.A) Normal conjunctiva B) Trachomatous inflammation – follicular C) Trachomatous inflammation – intense D) Trachomatous scarring E) Trachomatous trichiasis F) Corneal opacity. Credit: WHO.



2.2.1 Global Prevalence of Trachoma – the numbers

Trachoma is currently endemic is 57 countries with the greatest prevalence of disease in Africa, but also includes countries in Asia, and the Western Pacific. Active trachoma affects mainly women and children, and trachomatous scarring (TS) affects women three times more than men. This is likely due to their increased interaction with infected children [1,15,16], leading to greater exposure. Women account for 60% to 85% of all cases of trichiasis. Survey data also show that women are at higher risk than men for corneal opacity, trichiasis, and scarring, thereby indicating a great gender disparity within infection prevalence worldwide [15]. In 2003, WHO estimated that 84 million individuals suffer from active trachoma, while 7.6 million are left blind as a result of corneal opacity due to trachoma infection. Population based

surveys and studies have been conducted in order to map the global distribution of trachoma, as well as to obtain an accurate prevalence of trachoma [1]. With intervention efforts and epidemiological research, the most recent data suggest that trachoma is responsible for the visual impairment of 2.2 million people, of whom 1.2 million are irreversibly blind[17].

Chlamydia trachomatis infection is difficult to diagnose accurately [7]. The clinical exam can reveal ambiguity as no two trachoma graders are the same, and there is no gold standard test for examination [5]. Through a latent class analysis (LCA), See and colleagues estimated the sensitivity and specificity of laboratory and clinical diagnostic tests. The focus of the study was TF, TI, and PCR in the absence of a gold standard for trachoma. When compared to an ideal, true gold standard, TF clinical exam scores over estimated prevalence of trachoma infection, and was poorly specific. Poor specificity may lead to unnecessary treatment due to false positives. However, TF examination is advantageous since it is inexpensive, sensitive, and yields an instant clinical exam score.

TI is not currently used by WHO in trachoma treatment protocols and recommendations when determining thresholds for mass drug administration (MDA) particularly because TI is often overestimated. This result may be because TI diagnosis is associated with the presence of scarring and redness, and these clinical manifestations may not be due to active trachoma infection. See and colleagues observed higher specificity in TI than in TF, at 88.3% and 36.6% respectively. Studies have also suggested that TI correlates more strongly to PCR results [18,19], and TI patients also exhibit a higher bacterial load by quantitative PCR [20]. The underestimation and lack of sensitivity of TI prevalence seen in this study may be due to infection kinetics, as TI clinical signs clear up sooner than TF clinical signs [20]. The differing kinetics between the two clinical classifications demonstrate that clinical signs alone are unlikely

to produce an ideal representation of the current trachoma prevalence. Over- and under-estimations may steer elimination programs in the wrong direction and may result in over spending of funds, or the inability to reach all populations at risk.

In low trachoma prevalence areas, it is common to find TF clinical signs without trachoma infection. As stated previously, this could be due to lingering clinical signs after infection has cleared, Moreover, in low prevalence communities, the association between the clinical signs of TF and detectable infection of *Chlamydia trachomatis* is weak [21]. Studies have suggested that there are other, non-chlamydial bacterial agents that may be causing an inflammatory response, giving a clinical presentation similar to TF. Through a cross-sectional survey of children in a trachoma endemic community in Tanzania, Burton and colleagues investigated the relationship between TF clinical signs, *Chlamydia trachomatis* infection, and nonchlamydial bacterial infections by using the clinical exam criteria, polymerase chain reaction (PCR), and microbiologic cultures.

A TF diagnosis was found in 65 of the 473 children examined in the Burton study (13.7%), while *C. trahcomatis* infection was detected in 25 of the 473 children (5.3%). Statistical testing showed that there was no association between the presence of TF and the presence of *C. trachomatis* (95% CI = 0.40 – 3.64; OR = 1.21; p-value = 0.74). This weak relationship may lead to a number of individuals receiving antibiotics when trachoma treatment is not needed. It may also lead to overestimation of TF prevalence in the area and may not give an accurate portrayal of the actual prevalence of trachoma. Nonchlamydial bacterial organisms were cultured and identified from 305 of the 473 children (64.5%). A large variety of bacteria were identified within the study population. Many children infected with nonchlamydial bacterial organisms also exhibited co-infection with other bacterial organisms. Significant results were found for TF

positive children infected with *Streptococcus pneumoniae*, *Haemophilus influenzae* type-B or *Haemophilus influenzae* non-type-B when compared to TF negative children (p-values: 0.001, 0.008, and <0.001, respectively). Other studies have been conducted between 1962 and 1999 that show infection of *Streptococcus pneumonia* and/or *Haemophilus ssp* in trachoma endemic communities from Taiwan to Nepal [22,23,24,25,26,27]. However, these studies were conducted before sensitive PCR techniques were developed for the detection of *C. trachomatis*, possibly producing results with low sensitivity and specificity. Past studies have also shown that individuals with trichiasis (TT) and scarring (TS) are more likely to be infected by nonchlamydial bacterial pathogens. These results suggest that in communities with low levels of trachoma prevalence, it is possible that inflammatory signs similar to TF may be caused by other bacterial agents.

2.2.2 Economic Burden due to Infection

Trachoma is associated with great economic burden and loss of productivity due to its blinding and visually impairing effects. Since 1996 and the formation of GET2020, a number of studies have been performed in order to estimate the burden of disease through disability adjusted life year (DALY) measures, and have also estimated lost potential productivity figures in order to quantify the economic impact caused by trachoma [28]. Disability calculations can be compared to years of life with disability (YLD) and years of life lost (YLL). Depending on the availability of data, not all DALY calculations will include YLD and YLL estimates. Also, assumptions of employment and payroll status can lead to overestimations particularly for countries with substantial unemployment rates [28,29]. Alternatively, underestimation may result because many models do not account for the impact of trichiasis prior to visual impairment [30], non-workplace productivity losses, and time spent caring for blind individuals [28]. Also, studies

have assumed that blind individuals cannot be productive at all, or are much less productive than the 40% productivity level implied by the DALY weight [28,31,32]. With the caveat that these estimates may be uncertain, trachoma is associated with an estimated 5.3 billion USD in productivity losses due to globally impaired vision, and prevalent cases of trachomatous visual loss yield 39 million lifetime DALYs. The greatest burden of DALYs, both in one year and lifetime, is seen in Sub-Saharan Africa for both blindness and low vision. The total cost in productivity loss is likely to be an underestimate of the social cost of the disease.

Large gender disparities exist between men and women regarding trachoma. Survey data in the literature has consistently shown that women are at a much greater risk of trachoma infection than men, with female prevalence being two to four times greater than that in men. Trachoma prevalence is generally greater in women than in men due to their interaction and care for children that may be infected [1]. The majority of the burden measured by lifetime DALYs associated with prevalence falls on women, with 80% of DALYs found in women. This translates into greater economic loss estimated at over 2 billion USD per year in potential productivity among women in trachoma endemic regions [28].

2.3 Trachoma Prevention

Prevention of trachoma infection is driven by a public health initiative, known as the SAFE strategy. The goal of GET2020 is for the global elimination of trachoma by the year 2020. The acronym for the SAFE strategy represents four components aimed at the prevention and treatment of trachoma. In terms of treatment, surgery is used for trichiasis patients, and antibiotics are used for patients exhibiting active trachoma (TF/TI). In terms of prevention, facial cleanliness and environmental improvements are necessary in order to disrupt the cycle of trachoma transmission and infection. World Health Organization bases its treatment

recommendations on the prevalence of active trachoma as determined by clinical exams. Treatment strategies must be modified according to the baseline trachoma prevalence of the community [5,33,34,35]. Accurate trachoma surveillance is necessary in order to steer trachoma prevention and elimination programs.

2.3.1 Focusing on the 'A' in SAFE

The 'A' in the SAFE strategy is driven by World Health Organization recommendations to implement mass drug administration (MDA) in trachoma endemic regions. WHO currently recommends either tetracycline ointment or azithromycin for the control of trachoma. If the baseline prevalence of a district of TF in 1-9 year old children is 10% or greater in a district, antibiotic treatment is provided to all residents annually for three years. After the initial three years, a district survey is repeated. If prevalence is above 10%, MDA at the district level is continued. If prevalence is below 10%, the community level is assessed. If surveys show a community level prevalence of 5% or greater, treatment continues until prevalence is below 5%. When community level TF prevalence is below 5%, MDA is stopped. After antibiotic treatment has stopped, the prevalence of trachoma should be measured twice, at two 3 – year intervals according to World Health Organization recommendations, to verify that infection levels have not increased.

A number of studies have been conducted focusing on the antibiotic arm of the SAFE strategy. WHO recommends azithromycin administration for all children in endemic communities, except for infants under six months of age, who are given tetracycline, as part of the current trachoma control strategy. However, because mass drug administration (MDA) is distributed based on trachoma prevalence within the district or at the community level, it can be costly and results in many uninfected individuals receiving treatment. Studies have shown that

there is poor correlation between active disease and infection in patient. This fact has lead researchers to explore more targeted approaches for trachoma control [36]. Studies targeting antibiotics to households for trachoma control within The Gambia and Tanzania have shown that clinical signs are significantly more sensitive as an indicator for infection in the household than as an indicator for infection in the individual. Using model simulation, targeting treatment to households has the potential to be as effective as and significantly more cost effective than mass treatment when antibiotics are not donated. However, when antibiotics are assumed to be donated as is currently seen in trachoma control programs with Pfizer donations, MDA is projected to be more cost effective than a targeted strategy [36].

In theory, repeated treatment with antibiotics can reduce the prevalence of trachoma over time, and may even eliminate infection [35,37]. However, re-emergence is likely to occur in hyper-endemic areas [21,38,39]. The control of trachoma is particularly important in these hyper – endemic areas because of their susceptibility to re-emergence, as this stalls progress towards elimination. Some studies suggest that if there is an increase in coverage, fewer rounds of treatment would be needed. Therefore, elimination would be reached in a shorter time period [37]. In order to determine the efficacy of one round of mass drug administration, Lakew and colleagues observed the relationship of antibiotic coverage and treatment efficacy. By examining 40 villages in Ethiopia through cluster randomized – control trials, Lakew and colleagues examined the prevalence of trachoma at baseline, and at two and six months post treatment. With a mean coverage of 90.6% and analysis through multivariate regression models, Lakew and colleagues show that chlamydial prevalence was significantly associated with baseline infection and antibiotic coverage at two months post treatment. However, at six months post treatment, chlamydial infection was only associated with baseline infection. Mean baseline prevalence for

the 40 villages was 48.9% in children ages 1-5 years. Prevalence decreased significantly two months after treatment (to a mean of 5.4%, $p < 0.0001$), but then increased at the six month post treatment mark (to a mean of 7.9%, $p = 0.03$ when compared to two months post treatment).

The results found by Lakew and colleagues may have a number of explanations. Communities of high trachoma prevalence are more likely to experience residual infection after an incomplete mass treatment round [33]. Also, re-infection is likely to occur rapidly in areas with high trachoma prevalence because poor sanitation and hygiene factors may not have been addressed. Therefore, the cycle of trachoma transmission will continue post treatment [40], any benefit that antibiotic coverage and mass treatment would have in reducing trachoma prevalence may be canceled out by re-emergence due to high prevalence, and poor sanitation and hygiene. Re-emergence due to poor sanitation and hygiene serves as an argument that antibiotics alone are not sufficient for the global elimination of trachoma. Finally, not all susceptible children were included in this study. Since the study only included children from ages 1-5 years, this study did not include administration of tetracycline for those under 6 months, or oral azithromycin for those between 6 months and one year, and this may have contributed to a faster rebound in infection.

Adherence to tetracycline becomes an issue, as adherence to the topical ointment is less than azithromycin [41]. Azithromycin is distributed as a single dose annually to children over six months of age, whereas tetracycline eye ointment is recommended for children under six months and must be applied twice a day for six weeks in order to be effective in trachoma – endemic communities[40]. Tetracycline ointment is also difficult and unpleasant to apply to young children. With poor compliance, re-infection is possible due to exposure with untreated infants in the household level [37]. However, there are studies that do not show evidence that infants

increase the risk of re-emergent infection in households after MDA [42]. When West and colleagues controlled for confounding factors, children living with an infant had a reduced, but not statistically significant, risk of trachoma infection six months after treatment. However, the two studies employed two different populations with different baseline trachoma prevalences (5.9% in the West study versus a mean 48.9% in the Lakew study). Again, re-emergence may have been more likely in the Lakew study due to high trachoma prevalence, and poor sanitation and hygiene – factors that will continue the cycle of transmission. Also, West and colleagues did not examine compliance among patients using tetracycline, so compliance may have been better than expected.

The current recommendation for trachoma control via MDA over three years for districts with over a 10%, baseline prevalence of TF has been explored. Stopping MDA prior to three rounds of MDA may be more cost effective if monitoring indicates an absence of infection. Many individuals may exhibit the clinical manifestations of trachoma, but would test negative for infection. Using tests for infection would serve as more useful that using clinical signs for directing MDA efforts. Previous studies have shown that it is unclear how many rounds of MDA are necessary to obtain a prevalence of 5% at the community level, particularly for hyper – endemic communities [43,44]. If monitoring indicates an absence of C. trachomatis in the population, MDA could be stopped prior to the completion of three antibiotic rounds, therefore being more cost-effective and saving antibiotics for populations in greater need.

Studies in Tanzania and the Gambia suggest that districts with low trachoma prevalence, between 10% and 20%, and high antibiotic coverage may reach trachoma elimination within one round of treatment [45,46,47]. The PRET – Ziada Trial explored the possibility of ceasing MDA prior to three annual rounds of antibiotics. Studies were conducted by West and

colleagues in the Kongwa district of Tanzania in order to determine if MDA could be stopped before WHO's recommended initial three rounds of antibiotic treatment. Sixteen communities were selected, and eight were given the WHO recommendation of treatment, while the remaining eight were treating according to a cessation rule. According to the cessation rule, the eight villages would be exposed to yearly mass treatment if *C. trachomatis* infection prevalence was above five percent. Otherwise, MDA would cease for these communities and they would then be monitored for re-emergence. The cessation rule cut-off and monitoring strategy was based on the work of Lakew and colleagues observing re-emergence of trachoma in communities within Ethiopia [48].

West and colleagues sampled 100 children from each community that were five years of age or younger. The clinical trial took place between February 2010 and September 2011, and surveys were taken at the 6 month and 18 month marks during the study period. At each survey visit, a clinical examination of trachoma status was performed, as well as an eye swab of the conjunctiva of the right eye for polymerase chain reaction (PCR) analysis in order to determine the infection status of the individual. Additionally, the children were assessed for facial cleanliness and the presence of one of the following signs: ocular discharge on the eye, nasal discharge on the nares, cheeks, or lips, and flies landed on the face during a three second interval. These three signs have been used in previous studies by West and colleagues observing facial cleanliness and the risk of trachoma within families[49].

Results showed no statistically significant differences between the two groups in TF prevalence at any point during the study. At the 18-month survey, there was no statistically significant difference in the infection rates between the usual care communities and the cessation rule communities (2.9% versus 4.7%; p – value = 0.25). The results demonstrated that MDA

could not be stopped before the three year period of treatment based on the cessation of rule of infection prevalence less than five percent. After one round of MDA, active trachoma prevalence in both groups decreased to less than 10% from an estimated 20% at baseline. No community ceased MDA prior to the third round of MDA, which was unlike the results seen previously in the Gambia [45,46].

The cessation rule was chosen on the basis of previous studies in the Gambia, and has been described as a conservative rule by West and colleagues. If a less strict guideline was chosen for the cessation rule, MDA may have been able to be stopped earlier. However, there is no data to guide the selection of a cessation threshold. Re-emergence is also a possibility an ideal cessation threshold is not used, and studies have shown re-emergence to occur after antibiotic pressure is removed from a community [50]. Ultimately, West and colleagues concluded that using a rule for cessation of MDA based on infection status did not change the frequency of treatment within low prevalence communities [51].

Determining measures for indicating trachoma infection will help predict whether MDA could be stopped in endemic regions. By determining indicators for trachoma infection, as well as determining cut-offs for MDA, trachoma elimination programs will be able to alter their programs for the most beneficial and cost effective strategy at the community level. High risk signs (HRS) are defined by West and colleagues as clinical sign combinations that indicate trachoma prevalence over 20%. These combinations include: groups with any severe TI, groups with both TF and TI, and groups with any sign of inflammation indicating TI. Studies in Tanzania have shown that using trachoma high risk signs (HRS, as defined by West and colleagues) to determine the infection status of a community was not useful in predicting whether MDA could be stopped. High risk signs were predictive of infection within the

community, but they were not indicators of low infection rates [3]. From the studies by West and colleagues, new methods outside of clinical examinations and PCR are needed in order to assess community infection rates, and to ultimately determine threshold cut-off for MDA.

2.4 Diagnostics tools for *Chlamydia trachomatis* – genital and ocular studies

Trachoma diagnoses are generally made based on clinical observations. Laboratory diagnostic tools may be unaffordable or unavailable, especially in trachoma endemic areas. A number of laboratory diagnostic tools can be used such as microscopy, cell cultures immunoassays, and nucleic acid amplification. The micro-immunoflourescence test (MIF test) is generally considered the gold standard for genital Chlamydia disease detection [52]. However, analysis of MIF tests is subjective, leading to laboratory variation in MIF results [53]. Also, the specificity of the MIF test has been questioned due to cross reactivity between Chlamydia subspecies, leading to high rates of false positive results [54]. These difficulties called for more specific serological tests in order to overcome the limitations in the MIF test.

In assessing the various laboratory tests, Solomon and colleagues discussed the varying cost, sensitivity, specificity, technical complexity and processing time needed for each technique. PCR was found to be the ideal laboratory diagnostic tool due to its high sensitivity and high specificity, and its straightforward sampling requirements, which is a conjunctival swab [7]. See and colleagues demonstrate that PCR is an ideal form of measure for trachoma infection as its sensitivity and specificity are close to an ideal, gold standard [5]. However, PCR is shown to be one of the more costly tools available and is usually not available in trachoma endemic settings for community level assessments. TF is the WHO recommendation for the trachoma clinical exam, but results from the clinical exam and PCR laboratory diagnostics do

not always align. Therefore, there is a need for additional tools for surveillance, especially to monitor infection prevalence after MDA has ceased.

Enzyme immunoassays (such as ELISA) are more advantageous than PCR when compared in terms of technical complexity and cost. However, enzyme immunoassays have both lower sensitivity and specificity than PCR, leaving room for error in trachoma prevalence assessments. Also, enzyme immunoassays may be single antigen based, limiting the amount of antigens that can be observed per test, and therefore, increasing the number of tests to be performed in order to observe multiple antigens.

2.4.1 The multiplex bead assay (MBA) and Immunological Assays

The multiplex bead assay (MBA) is an immunological assay that quantifies antibody responses in median florescence intensity. The assay is most often compared to enzyme-linked immunosorbent assays (ELISA) to determine differences in sensitivity and specificity between the two tools. The two tools are similar in that both use the bound antigen to capture a soluble antibody. Detection of the captured antibody is done by a second, detection antibody. However, there are differences in the protocols of MBAs and ELISAs. The MBA uses a fluorescence system of detection in order to quantify responses. Also, ligands are captured onto microscopic, spherical beads in suspension, while an ELISA relies on the flat surface of a 96-well plate. Since ligands are captured onto individually classified beads, a variety of lingand – bound beads can be placed into each well of a 96-well plate, allowing for the observation of responses of a number of various diseases, and multiple antigen ligands specific to each disease within a small volume. Small volumes of blood or human sera allow for minimally invasive procedure for sample collection, such as using a finger prick for bloodspot collection and analysis. Therefore, multiplex bead assays have the potential to survey a large number of diseases in one assay, whereas multiple ELISA assays would be required to produce similar data. Though MBAs have potential to survey a multiple ligands simultaneously,

cross reactivity between ligands may reduce sensitivity and specificity, and produce artifacts in the data analysis [55].

The multiplex bead assay (MBA) has been used in a number of studies to detect and quantify antibodies found in biological fluids from animals and humans, and offers advantages compared to single antigen based enzyme-linked immunosorbent assays. First of all, the multiplex bead assay allows for the simultaneous collection of multiple data points from a single specimen sample. Multiple antigen-coupled beads can be analyzed in one assay thereby generating a plethora of data. This eliminates the single data point that would be obtained per specimen sample in an ELISA analysis. By using a 96 well plate format, up to 100 different antigen-captured bead classifications can be examined per well, producing 9,600 data points in one assay. This speed and volume of data produced by one MBA reduces the labor, time, and cost needed to produce the same results by using single antigen based ELISAs [8]. Multiplex bead assays are ideal for mass disease screenings of a particular region, since numerous antigens of various diseases can be tested at once. This strategy can help determine diseases of priority, those of high prevalence, and those that may have re-emerged over time. Multiplex bead assays have been studied for their potential use in disease surveillance overtime to determine the progress of disease intervention programs. MBAs may assist in determining the direction of programmatic interventions, as well as policy decisions regarding elimination efforts.

2.4.2 Serological Tools for Public Health Programs

Due to the large amount of data points that can be produced by MBA analysis, the serological tool has been studied for its possible uses in large scale surveillance programs for NTD control and elimination programs. The sensitivity of the MBA is similar to that of an ELISA, so similar results may be seen [56], however, MBA provides more data points for a number of disease specific antigens - up to 100 antigen coupled beads per well - and saves on

cost, labor and time. PCR tests are ideal for their high sensitivity and specificity, but are costly at $10 to $15 per test [7] For MBA, only one microliter of serum specimen is required per well, and is estimated to cost between $90 and $95 to run one, 96 well-plated assay with numerous antigen – coupled classifications [8]. Due to the difficulty of standardizing the clinical exam for trachoma, and the fact that clinical signs persist after infection is cleared, there is a need for additional serological tools for surveillance of trachoma infection, even after MDA has ceased. Additional serological tools would assist with the monitoring of disease prevalence, would serve as a method of monitoring re-emergence, and would assist in determining programmatic policies and programs for NTD elimination.

Studies have been conducted observing MBAs within populations that are enrolled in NTD treatment studies. Moss and colleagues suggested through their studies of lymphatic filariaris in Haiti that MBA can monitor MDA program effectiveness overtime, and observe antibody positivity and response to drug regimens. Again, this study also demonstrated the advantages of using a multiplex assay over a single antigen based ELISA to observe a number of diseases of co-infection. [8].

Studies have particularly examined pgp3 and CT694 for trachoma infection using the multiplex bead assay [2]. As stated previously, a number of studies examining pgp3 and CT694 antigen based ELISA were for the observation of genital Chlamydia. Ocular Chlamydia has not been explored as extensively as the genital form. With elimination goals in mind and the review of the previous literature, the two antigens in conjunction with the MBA may also be useful for ocular Chlamydia.

By examining a cohort of children from trachoma endemic villages in Tanzania, Goodhew and colleagues observed IgG responses by using a MBA with pgp3 and CT694 antigen

coupled beads. These responses were then analyzed against the clinical manifestations of active trachoma. A sample set from the United States served as a control. However, the samples were unidentified and, without a patient history, it is not possible to determine the true disease status of the tested population. As seen in other studies, it is possible that individuals in the control group may produce a positive response if the individual acquired chlamydial infection through vertical transmission. In this study, Goodhew and colleagues were able to detect and correlate antibody responses with the clinical exam outcomes and PCR status on the community level and the individual level. Individuals that showed a higher pgp3 and CT694 antibody response also had higher trachoma prevalence via clinical exam outcomes. Of children without evidence of active trachoma, many still showed a positive antibody response for *C. trachomatis*. These results most likely represented children that have been previously infected with trachoma [9]. This study revealed that multiplex bead assays may be used for trachoma specifically, and not just for genital Chlamydia surveillance.

2.4.3 Candidate antigens: MOMP and CPAF

The major outer membrane protein (MOMP) of *C. trachomatis* is a major variant surface protein and is suggested to play a role in protective immunity of the bacteria. MOMP has been examined in vaccine studies to combat chlamydial infections of various subspecies, as well as antibody based diagnostics tools, such as ELISA [57]. MOMP served as a candidate antigen for antibody based diagnostic tools, however, studies suggested that other antigens, such as pgp3, had higher levels of sensitivity and specificity when compared to MOMP [58]. Also, MOMP is found in the Chlamydia genus, but is not species specific. Therefore, false positives for other species of Chlamydia may be detected when using MOMP based serological tools.

Chlamydial protease or proteasome-like activity factor (CPAF) has been suggested produce a number of different effects in the host cell, such as the cleavage of host cell structures. CPAF has been suggested to be an important virulence factor of the bacteria, and studies have shown that CPAF degrades specific host proteins [59]. CPAF is also suggested to be integral in evading the host immune response [60]. Skwor and colleagues have suggested that, among individuals with trachoma infection, IgG antibody responses to CPAF – based quantitative ELISAs are likely to be a marker for inflammatory trachoma and trichiasis disease, as responses to CPAF were significantly higher when compared to the control specimen [61]. As seen with MOMP, CPAF is found in the Chlamydia genus, but is not species specific. Therefore, false positives for other species of Chlamydia may be detected when using CPAF based serological tools.

2.4.4 The trachoma antigens pgp3 and CT694

Wills, Frikha – Gargouri, and their respective colleagues suggested that the trachoma antigens, pgp3 and CT694, are ideal for the detection of *C. trachomatis*, and both have the potential to be used in serological tools for the surveillance of *C. trachomatis* infection over time. This is particularly important for trachoma, as monitoring prevalence after MDA has ceased will help determine programmatic policies to reach WHO's goal of global elimination of trachoma.

Identifying ideal antigens to serve as indicators for infection is an important task that is necessary for MBAs. For trachoma, pgp3 has been shown to be an ideal candidate for MBAs throughout previous studies. The *C. trachomatis* antigen pgp3 has been determined to be a useful antigen for the detection of infection via serology based assays. The pgp3 protein is a *C. trachomatis* specific antigen that is rarely found in other chlamydial isolates, such as *C.*

pneumoniae. The sequence is also highly conserved between strains, and a small percentage (less than 1%) of divergence is observed [58]. This prevents cross reactivity between antibodies of the two subspecies and will decrease the possibility of false positives.

Wills and colleagues suggested that a pgp3 antibody ELISA is both sensitive and specific for the analysis of genital Chlamydia infection. An immunoglobulin G (IgG) ELISA based on the antigen, pgp3, was evaluated against three other, commercial ELISAs derived from the major outer membrane protein (MOMP). Wills and colleagues examine two cohorts, one Chlamydia positive population and one Chlamydia – negative population, in order to determine the sensitivity and specificity of the four ELISA designs. This study also helped to show the superiority of pgp3 over MOMP for detecting chlamydial infection. The pgp3 antibody ELISA was significantly more sensitive than the three commercial ELISAs, with sensitivity of 57.9% compared to 44 – 49% sensitivity in the commercial ELISAs (p value <0.003; 95% CI: 52.7% to 62.9%). Specificity of the pgp3 ELISA was 97.6%, but was not significantly different from the specificity of the commercial assays, ranging between 96.0% and 99.0% [58].

As the design of this study was to examine genital Chlamydia, the Chlamydia – negative population was a cohort of children ages 2 to 13 years of age. Although children are unlikely to have Chlamydia exposure, it is possible through consensual sexual intercourse, ocular trachoma, vertical transmission from the mother, and sexual abuse. For children in the study that tested positive, patient profiles revealed a portion of the children coming from trachoma hyper-endemic regions [58]. Pgp3 protein is found in both ocular and genital Chlamydia; therefore, there remains the possibility that a positive test may indicate either genital or ocular trachoma.

Similar studies have been designed in order to determine the diagnostic value of CT694 based ELISAs for the detection of *C. trachomatis*. Similarly to pgp3, CT694 is a species specific

protein to *C. trachomatis* and is not found in other chlamydial subspecies [62]. Again, this prevents cross reactivity between subspecies, and reduces the possibility of observing false positives. Frikha – Gargouri and colleagues evaluated the diagnostic value of a CT694 ELISA. Comparisons against peptide based ELISAs (pELISA) and the MIF test in order to determine sensitivity and specificity.

The CT694 antigen based ELISA performed better than the MIF and the pELISA. When results were compared to the MIF test results, no high correlation was found and the R – squared value was 0.387. Frikha – Gargouri and colleagues observed a large number of sera that exhibhited high results in the ELISA test, but low results in the MIF. The greatest performances of the CT694 ELISA were obtained when compared to the species specific pELISA. Sensitivity and specificity were determined to be 85% and 87% respectively. Due to its high specificity and sensitivity when compared to the MIF and pELISA tests, the CT694 ELISA will reduce the number of false positives in a sample size, and will also assist in reducing overall overestimation of disease prevalence. Correlations between the CT694 ELISA and the MIF or the pELISA were generally low. This is due to the fact that all three tests are inherently measuring antibody responses directed towards different antigens. The CT694 ELISA, MIF, and pELISA observe antibodies against CT694, antibodies against the chlamydial elementary bodies, and antibodies against a specific peptide of MOMP, respectively. Past studies have shown these similar, low correlations [54].

From the results of these studies, the use of pgp3 and CT694 together in serological assays have proved to be useful for surveillance and monitoring of trachoma prevalence when used in conjunction with the multiplex bead assay. Both pgp3 and CT694 are ideal for the detection of *C. trachomatis*. The two antigens have the potential to be used in serological tools

for the surveillance of *C. trachomatis* infection over time, and may serve as candidates for monitoring trachoma prevalence after mass drug administration has ceased. Monitoring prevalence after MDA has ceased will help determine programmatic policies to reach the goal of global elimination of trachoma.

2.5 Conclusion

The literature shows a number of quality studies examining trachoma clinical exams and serological exams. More research is needed in order to observe trachoma infection dynamics with the MBA in trachoma endemic regions. As seen in the literature, many children may exhibit a high IgG response without the clinical manifestations. Immunoglobulin A (IgA) has served as a marker for recent infection, and an IgA based MBA may assist in distinguishing between past and recent trachoma infections. Distinguishing between recent and past infections will provide a more holistic picture of trachoma transmission dynamics, incidence, and may also assist in programmatic direction for MDA.

Chapter 3: Manuscript

Title

Assessment of IgA responses to *Chlamydia trachomatis* antigens among children from trachoma - endemic communities

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Contribution of the student

Ms. Morgan was responsible for the analysis of the collected bloodspots by using the multiplex bead assay in order to analyze IgA antibody responses to the trachoma antigens, pgp3 and CT694. She was also responsible for the analysis of the collected data through statistical analyses, and the production of the graphs and tables displayed in this thesis.

Abstract

Trachoma, caused by the bacteria, *Chlamydia trachomatis*, affects millions of individuals worldwide, and is a leading cause of visual impairment and blindness. Nucleic acid amplifications tests are being used to confirm infection, but tests are expensive and are not routinely available in trachoma – endemic areas. After the introduction of mass drug administration (MDA), trachoma infection diminishes, but clinical signs may continue for a long period of time after infection prevalence declines, requiring new tools to determine if MDA is still warranted, and to carry out surveillance when MDA has stopped. We have previously shown that IgG response against the trachoma antigens, pgp3 and CT694, were associated with ocular pathology and active infection; however, many children with IgG antibody lacked evidence of either pathology or infection, indicating past exposure. In this study, we analyzed IgA responses to pgp3 and CT694 to determine if these provided a better marker of recent infection than IgG responses. Bloodspots were analyzed from a cohort of Tanzanian children (n=155) using an antibody – based multiplex assay to determine associations between IgA antibody responses, infection status, and ocular pathology. A second set of bloodspots were analyzed from a cohort of Tanzanian children (n=173) using an antibody – based multiplex assay to determine the kinetics of IgA antibody responses over time after MDA. Ocular swabs were analyzed for presence of *C. trachomatis* infection using Amplicor®. Antibody responses to both antigens were associated with infection status as determined by PCR. Only pgp3 antibody responses were significant when observing normal and TF/TI ocular pathology (pgp3 $p = 0.0207$, CT694 $p = 0.4691$). For children without evidence of trachoma or infection, 23% tested IgA-positive to pgp3, and 6% tested IgA-positive to CT694. These results suggest that IgA responses are not specific indicators of active infection. The results of the kinetics of IgA responses show a significant decline in both antigens six months after treatment for most age groups. Further studies with larger sample sizes are required to verify the findings of the kinetics of IgA antibody responses.

Introduction

Trachoma, caused by the bacteria Chlamydia trachomatis, is an ocular, neglected tropical disease causing severe morbidity and disability across the globe. Trachoma causes an estimated 3.8 million cases of blindness and 5.3 cases of low vision [1,2] in Africa and Southeast Asia. The disease is transmitted by direct personal contact, and also can be spread by flies that have come into contact with an infected person. If left untreated, repeated trachoma infection may progress from active infection, defined as, trachomatous inflammation – follicular (TF) or trachomatous inflammation – intense (TI), to scarring of the inside of the eyelid. Trachomatous trichiasis (TT), distinguishable by the turned-in eyelashes, results in damage to the cornea due to scratching from the eyelashes, and can lead to irreversible blindness caused by corneal opacity (CO).

Elimination efforts encompass The World Health Organization's (WHO) Global Elimination of Trachoma by 2020 (GET2020) program, and are based on the components of the S.A.F.E. strategy. **S**urgery is used for trichiasis patients in order to prevent blindness, **a**ntibiotics are used as treatment for patients with active infection (TF/TI), **f**acial cleanliness is promoted to prevent the spread of infection from person to person, and **e**nvironmental improvements are through sanitation and hygiene are implemented in order to interrupt transmission. Pfizer has donated over 225 million doses of Zithromax (azithromycin) through distribution by the International Trachoma Initiative (ITI) (www.iti.org).

As elimination programs reduce trachoma prevalence over time, defining programmatic endpoints becomes a priority. Clinical signs of trachoma may persist for individuals clear of active infection [3]. It is also possible that similar ocular pathology may be present in low prevalence settings, as inflammation may be caused by non-Chlamydial bacteria [4]. Tests for active infection by Polymerase Chain Reaction (PCR) are available, but are costly. Currently, the

WHO endpoint for antibiotic use is a follicular trachoma (TF/TI) rate of less than 5% in children under 10 years of age. However, clinical exams can be difficult to standardize between graders [5,6,7]. Therefore, alternative approaches to monitor trachoma prevalence over time are necessary.

Currently, antibody based tools are being investigated to explore their potential contributions in defining endpoints for neglected tropical disease (NTD) surveillance. Investigation of their utility for policy and programmatic decisions has so far included NTDs such as lymphatic filariasis, onchocerciasis, as well as schistosomiasis [8,9,10]. Antibody tests are also more cost effective when compared to the currently used polymerase chain reaction (PCR) technique. Previous studies have explored antibody assays for Chlamydia infection [11], and investigation of antibody assays for monitoring trachoma programs has recently begun [2]. Previous studies have also explored the sensitivity and specificity of various serological tools, and their capacity for evaluating trachoma transmission, defining endpoints of trachoma infection, and monitoring trachoma infection after mass drug administration (MDA) has ceased. Studies have also shown that a large percentage of children have detectable antibody responses, but exhibit no clinical signs, indicating prior infection [2]. This shows that there is a need for serological tools for measuring incidence or recent transmission. This study explores the contribution of the IgA antibody responses in order to determine indicators for recent trachoma infection.

Determining more robust endpoints for trachoma programs is a priority for elimination since the clinical signs may persist in the absence of active infection. Cost effectiveness of testing for active infection also remains a priority in large scale sample surveys to determine prevalence. As state previously, PCR tests are costly, and antibody tests may serve as a more cost effective alternative to monitoring trachoma prevalence after mass drug administration (MDA) has ceased.

Some antibody tests, such as the multiplex bead assay, have the capacity to assess a number of diseases in one analysis, and can also measure antibody responses for a number of individuals in one assay. Therefore, large scale surveys may be performed for large populations and can also integrate a number of NTDs for mass prevalence screening. Exploring IgA as a potential marker for recent infection will help determine the incidence of trachoma infection in order to steer elimination programs. Since IgA responses are elevated during a first infection, and decrease rapidly over time after the first infection, increased IgA antibody responses may serve as an indicator of recent infection in a population. Determining recent infections in a population can help differentiate past and recent infections, and will give public health practitioners and researchers a more accurate picture of trachoma transmission, as well as the progress and effectiveness of MDA efforts. Also, after MDA has ceased, immunological assays can serve as a cost effective measurement of trachoma infection. Negative IgA results would indicate that there are no recent infections of trachoma within the population, and the community has reached elimination.

The purpose of this study is to observe the associations of IgA antibody responses to two trachoma antigens, pgp3 and CT694, to ocular pathology, PCR positive results, and age, and to also observe the distribution of trachoma by village after MDA. Also, this study aims to examine the kinetics of IgA antibody responses to pgp3 and CT694 over time, and determine factors in which responses may decrease after MDA.

Methods

Two, separate data sets were used for this study. Studies were conducted in the Kongwa district (Dodoma region) of Tanzania as part of ongoing clinical trials to evaluate the impact of alternative models of community wide treatment with azithromycin, as well as determine the

kinetics of antibodies in antigen based assays. The trachoma prevalence in Tanzania can be seen by region in Figure 2. One study population was a subset of the four village populations as described by Goodhew and colleagues (n = 155). The study population for determining the kinetics of antibody responses overtime originated from untreated populations in the Kongwa district (Dodoma region) of Tanzania. Children ages 1 – 6 years of age were examined at baseline and at 6 – months post treatments via clinical examinations, and bloodspot samples and conjunctival eye swabs for PCR analysis were collected, as described elsewhere. A total of 173 children were examined at both baseline and the six month post treatment period. Parents or guardians provided written informed consent for children participating in the study. The study was approved by The Institutional Review Board of the Johns Hopkins University School of Medicine (Baltimore, MD) and the Tanzanian National Institute for Medical Research. Control samples were included in the analysis from United States samples. De-identified serum samples from a population of 123 children under the age of 6 years from the United States were collected as part of an IRB approved blood lead study. United States samples were used as negative controls and to establish a cutoff value for positivity in the multiplex assay for both trachoma antigens.

All materials and methods have been described elsewhere by Goodhew and colleagues concerning initial selection of CT694 and pgp3 antigen, and expression and purification of chlamydial recombinant proteins. Measurements of antibody responses by ELISA were performed as described by Goodhew and colleagues. However, plates were developed with mouse anti – human IgA (Zymed) at a 1:400 dilution. Total IgA was detected with 50 ng of biotinylated mouse anti-human total IgA (Zymed) and streptavidin (HRP). Plates were read on a Molecular Dynamics UVMax kinetic microplate reader (Sunnyvale, CA) at an absorbance of 405

nm. All other preparations were performed as stated by Goodhew and colleagues.

Statistical analysis was conducted using GraphPad Prism 5.03 (GraphPad Software Inc., La Jolla, CA) and Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA). Specificity and sensitivity were calculated for both antigens by receiver operator characteristic analysis. Comparisons of clinical trachoma, PCR results for infection, and age groups were calculated with confidence intervals (CI) of 95% using Mann-Whitney tests and Wilcoxon matched pairs signed rank tests to generate p-values. Linear regression was used to determine the best fit line and slope of antibody decline for each age group of participants in the kinetics study.

Results

Receiver operator curves (ROC) were generated for each antigen using finger prick sera samples from 122 children from the United States and blood spots from 10 infection PCR positive children from Tanzania. For pgp3, a MFI-BG value of 11 was established as the threshold value for indeterminate IgA antibody responses and 120 as the threshold for positivity. Values for pgp3 between 11 and 120 were defined as indeterminate. For CT694, a MFI-BG value of 14 was established as threshold for indeterminate response, and 43 as the threshold for antibody positivity. Values for pgp3 between 14 and 43 were defined as indeterminate.

IgA response survey study

Nine out of 10 PCR positive children (sensitivity 90%) were positive in each assay. The PCR positive Tanzanian sample that was not detected in the antibody assay was negative for both pgp3 and CT694. Since PCR data were not available, specificity was determined by using multiplex values of the presumed negative US control children. For pgp3, one presumed negative child was positive (99% specificity) and one child was positive for CT694 (99% specificity). For the US children, only one child was positive for both antigens. IgA antibody responses are

plotted by country in Figure 3. The median antibody response of Tanzanian children was higher than children from the US for both (A) pgp3 and (B) CT694, and was significantly greater than the median of the US samples (pgp3 p-value <0.0001; CT694 p-value <0.0001).

IgA antibody responses for both antigens are plotted by village in Figure 4. The highest median antibody response was seen in village 0401 for both pgp3 and CT694. The lowest median antibody response was found in village 1001 for both antigens.

IgA antibody responses are plotted by trachoma clinical and PCR status in Figure 5, and are shown numerically in Table 2A and 2B for IgA antibody positivity for pgp3 and CT694, respectively. Antibody responses to both antigens were associated infection status, but only pgp3 showed an association between antibody responses and ocular pathology. The median IgA antibody response for children with normal ocular findings (a TF/TI score of zero) is lower than those with either observed ocular disease (a TF/TI score of two), but an association between IgA antibody responses and ocular pathology was only seen in pgp3. IgA antibody responses to CT694 were not significantly higher in individuals with ocular pathology when compared to those without pathology (pgp3 $p = 0.0207$ and CT694 $p = 0.4691$). The same associations were observed when children with normal findings were compared to children with a TF/TI score of one (pgp3 $p = 0.0200$ and CT694 $p = 0.4510$). When children with a normal ocular exam were compared to PCR positive children, antibody responses were associated with infection status for both antigens (pgp3 $p = 0.0022$ and CT694 $p = 0.0031$). All but one PCR-positive individual had a positive antibody response to pgp3, and all but two individuals had a positive antibody response to CT694. In Figure 3, it is also important to note that a large proportion of children with no evidence of ocular pathology had elevated pgp3 and CT694 responses (44% above cutoff for both antigens).

IgA antibody responses to pgp3 and CT694 are shown by age groups in Figure 6. Median antibody responses to both antigens increased with age, and the highest proportion of positive children are seen in the 6–9 year old age group. For pgp3, children under three responded significantly less than the other two age groups ($p=0.0154$ for 3 to <6 and $p= 0.0005$ for ≥6). For CT694, responses of children under three were similar to those of children in the 3 to <6 age group, but were significantly less than the ≥6 age group ($p=0.0503$ for 3 to <6 years and $p=0.0004$ for ≥6 years). Most of the antibody-positive children under age 3 years had a positive infection result via PCR analysis (indicated in red) or trachoma ocular pathology (TF/TI score of 2, indicated in green), while those with neither were more likely to be antibody negative for both antigens.

IgA Kinetics Study

For children enrolled in the IgA kinetics study, bloodspot samples were obtained at baseline and at 6 months post treatment following trachoma MDA. Median responses are plotted as median florescence intensity minus background (MFI – BG) at baseline by clinical trachoma score for A) pgp3 and B) CT694 in Figure 7. PCR positive children are indicated in red. At baseline for previously untreated children, IgA antibody responses to both antigens were associated with infection status and ocular pathology. The median IgA antibody response for children with normal ocular findings (a TF/TI score of zero) is significantly lower than those with observed ocular disease (a TF/TI score of two) for both pgp3 and CT694 (pgp3 $p < 0.001$ and CT694 $p < 0.001$). Similar differences were observed when children with normal findings were compared to children with a TF/TI score of one (pgp3 $p = 0.001$ and CT694 $p = 0.005$). When children with normal eye exam were compared to PCR positive children, antibody responses were highly associated with infection status for both antigens at baseline prior to

treatment (pgp3 p <0.0001 and CT694 p <0.0001). These results are expected as the children have not yet been treated for trachoma infection at baseline.

Antibody prevalence by age at baseline is shown in Figure 8A and 8B for both pgp3 and CT694, respectively. Cut offs for positivity were based on analyses of US samples as described previously. Prevalence increased with age for both pgp3 and CT694, and was highest in five and six year old children. By age six, 93% of children were antibody positive to at either pgp3 or CT694.

After treatment, antibody responses decreased for both pgp3 and CT694, comparing paired, baseline and six month post treatment medians, stratified by age. The p-values for both pgp3 and CT694 for each age group of antibody positive children are shown in Table 3, and indicate the significance of the change in mean antibody responses between baseline and six months post – treatment for children that were antibody positive at baseline for each age group. All ages groups showed significant differences in antibody responses from baseline to six months post treatment for both antigens, with the exception of 2 year old children, and 3 year old children for CT694.

At six months post treatment, IgA antibody responses decreased across all ages, as shown in Figure 9 for both pgp3 and CT694, respectively; however, few children reverted to seronegative status. The mean rate of decline in antibody for antibody positive children decreased with age. The greatest rate of decline for pgp3 was seen in the one year old children, while a lesser decline was seen in the six year old children (slope = -325.2 for one year old children, and -145.9 for six year old children). A similar trend in the rate of antibody response decline is seen in CT694 among antibody positive individuals (slope = -35.85 for one year old children and slope = -31.34 for six year old children). The rate of decline of IgA antibody

responses decreased between the extreme age groups, which may serve as an indicator of multiple infections of trachoma within the older population, as IgA antibody responses persist longer in human sera and blood after multiple infections. Discrepancies in slope declines were observed in children between the ages of two and five. Low samples sizes may have contributed to the variance in slopes. Also, since IgA is less prevalent in the blood, there is a high variance for IgA antibody positivity. The large variance in responses, in conjunction with a low sample size, does not produce an ideal normal distribution to compare the mean antibody responses from baseline to six months post treatment (data of remaining slopes not shown).

Discussion

Sensitive surveillance tools are necessary in trachoma endemic communities in order to determine whether or not MDA should cease. Within low resource settings, PCR data is often unavailable or not cost effective to support decision-making by endemic countries. The use of serological tools to detect antibody responses to trachoma antigens may provide an alternative to clinical exams and PCR analysis as indicators of interruption of transmission. The absence of an IgA antibody response might indicate an interruption of transmission in formerly endemic areas, which may in turn lead to the decision to MDA. In principle, an absence of IgA responses would indicate no recent infections, indicating elimination of trachoma incidence and interruption of transmission. The use of serological markers has been shown to be useful in detecting exposure to *C. trachomatis* in the context of genital infections, and has been recently explored for ocular infections as well [2]. In this study, we used serological markers to screen for IgA antibody responses and their relationship to ocular infections as assessed by PCR and clinical disease. Two antigens, pgp3 and CT694, were selected for the multiplex assay after an extensive literature search, and previous studies using the two antigens in the context of ocular *C.*

trachomatis. Two different communities were used for the purposes of this study. Bloodspot eluates were screened from Tanzanian children with two chlamydial antigens to measure IgA antibody responses to trachoma antigens after MDA and compared results to clinical exam and PCR analysis data. A second study screened bloodspot eluates from Tanzanian children before treatment and six months after treatment to determine the IgA antibody kinetics.

IgA response survey study

At the community level, communities with higher trachoma prevalence also had higher pgp3 and CT694 antibody responses. Village 0401 had the highest median levels of pgp3 and CT694 antibody along with the highest numbers of positive clinical exams. Village 1001 showed lowest prevalence of antibody positivity for both pgp3 and CT694 and lower trachoma prevalence. Village 1501 had lower antibody prevalence than Village 1602 for pgp3, but a slightly higher response to CT694. Therefore, community antibody prevalence is not concordant for the two antigens. This discrepancy shows that communities that may show positive results for pgp3 may not necessarily produce a positive response for CT694, an argument for the use of two or more antigens.

IgA responses to the two trachoma antigens differed significantly. For samples from children that exhibited only infection (n=7), 86% and 43% were IgA-positive for pgp3 and CT694, respectively. Of the 3 children exhibiting pathology and infection, 2 had a positive IgA response to pgp3, but none were positive for IgA to CT694. Of samples from children that exhibited only ocular pathology (n=20), 55% and 5% were IgA-positive for pgp3 and CT694, respectively. For children without evidence of trachoma or infection (n=125), 23% tested IgA-positive to pgp3, while only 6% tested IgA-positive to CT694. These results suggest that pgp3 has greater sensitivity for the detection of trachoma than CT694.

Of children with normal clinical exams, there were many with positive antibody responses. These likely represent children with previous infection, and similar patterns are seen in IgG analysis [2]. These results suggest that IgA responses are not specific indicators of active infection, but additional study of the kinetics of trachoma antibody responses is needed.

Lack of antibody in young children may be indicative of interruption of transmission and protection by the SAFE strategy, as shown for responses to pgp3 and CT694 in children less than three years of age in communities where MDA was provided. Antibody responses increased with age, and statistically significant differences in response were observed for both age group comparisons in pgp3, and one age group for CT694. Further analysis and characterization of pgp3 and CT694 are necessary in order to determine the reason for discrepancies between antibody responses.

There are several limitations to consider in the context of this study. First, all villages have been treated by MDA prior to sample collection, so the baseline antibody responses are unknown for each antigen. Also, because samples were collected at only one time point, our understanding of how each child's antibody and disease status changed over time is limited. The longitudinal kinetics study in the second portion of this discussion evaluates the change over time regarding antibody responses. Antigens were chosen based on literature specific to genital chlamydial infections [15] and previous studies with ocular trachoma [2]. Due to the limited available data of trachoma and multiplex assays, it is unclear that these antigen choices are the most appropriate in terms of recent trachoma infection. As stated previously, individuals that were antibody positive for pgp3 were not necessarily positive for CT694. Therefore, further studies of the two antigens and their differences are required to understand antigen mechanics, and the purpose of this decline in responses.

In summary, though having low levels in the blood, IgA responses to the trachoma antigens, pgp3 and CT694, are detectable using the multiplex bead assay. The antibody response is present in children with a PCR positive result (active infection) or signs of the ocular disease. Elevated antibody responses are also seen in children with a TF/TI score of 0 and those that are not PCR positive. Therefore, IgA antibody responses are not specific indicators for active infection for trachoma. Further analysis in the kinetics study displays the level of antibody response over time after treatment.

IgA Kinetics Study

For baseline samples, higher antibody responses were associated with ocular pathology and infection status. These results are expected. At baseline, antibody positivity increased with age. By age six, 93% of individuals were antibody positive for either pgp3 or CT694. Increasing antibody prevalence with age may be due to repeated infections, as IgA levels, though at lower levels in the blood, tend to persist for longer periods of time with repeated infections. The lower than expected antibody prevalence for children at age two for both pgp3 and CT694 may have resulted due to the small sample size of antibody positive children within the age group. A larger study population is required to observe the kinetics of IgA antibody responses by age group.

Post treatment, median antibody responses were compared between baseline and six months post treatment and stratified by age. Individuals that were antibody positive at baseline for pgp3 were not necessarily positive for CT694, confirming the results of the cross sectional study. This reinforces the need for further studies on the immunological differences between the two antigens. All age groups suggest a significant decrease in antibody responses six months after treatment for both antigens, except for individuals at age two for both antigens, and individuals at age three for CT694, but both groups had smaller numbers of children tested.

The slope of decreasing antibody responses between baseline and six months post treatment is greater in one year old children than in six year old children. Younger children may experience a greater IgA antibody decline than older children, since the infection being treated may be the child's first infection, whereas, in older children, IgA antibody persists for longer periods of time due to re-infection. This pattern was seen for both pgp3 and CT694.

Comparing antibody survey results to those found by Goodhew and colleagues showed that antibody responses were significantly lower for IgA than IgG. This is expected, as IgG is more predominant in the blood. Also, IgG antibody responses were significantly greater in individuals with ocular pathology for both pgp3 and CT694. For IgA, only pgp3 demonstrated antibody responses were significantly greater for individuals with ocular pathology [2]. This insignificant decline may be due to low IgA antibodies present in the blood, and the low responses seen in CT694. Further research of pgp3 and CT694 is needed in order to determine their antibody reactions, and the reason for declined responses in CT694.

In summary, within the scope of the kinetics study, IgA antibody responses were shown to decrease after treatment overall for the trachoma antigens, pgp3 and CT694. Insignificant associations with age groups between the median baseline antibody response and the median six month post treatment response may be due to low sample sizes. By stratifying by age to eliminate confounding, the rate of decrease in antibody levels between baseline and six month post treatment decreased as age increased, with a smaller rate of decline seen in six year old children versus one year old children. Therefore, IgA responses do decline after treatment. Antibody responses also decrease throughout time by antigen, and by age.

Limitations for the kinetics study include a sufficient sample size. For analysis, age was stratified in order to eliminate confounding due to repeated infections with age. However, this

decreases the observed sample populations within each observed group. A larger sample size would provide more power for the analysis. A larger sample size would also provide a higher power for linear regression analysis of the rate of antibody response decline over time, providing more accurate measures of slope for each age group. The US samples that served as negative controls for both studies are unidentified, and the travel and medical history of the samples are unknown. Therefore, the health status of the children is unclear, and there is a possibility that Chlamydia infection was acquired at birth maternally for children that exhibit elevated antibody responses [22], [23].

The multiplex bead assay may be a valuable antibody-based tool for trachoma surveillance, however, further investigation of pgp3 and CT694 are required in order to fully understand the differences between the two antigens. Expanding the use of the multiplex bead assay can allow for the analysis of antibody prevalence for a number of antigens corresponding to a number of diseases in one analysis. This allows for mass screening of multiple NTDs within a given community. Mass screenings through serological testing would prove to be effective and efficient in communities endemic to multiple NTDs, versus using multiple, disease specific surveillance methods. For trachoma surveillance, multiplex bead assay analysis may be valuable in determining the median fluorescence intensity (MFI) that represents the cut off for antibody positivity. Determining positivity in the serology will provide a more accurate picture of the current trachoma prevalence. Also, multiplex bead assay analysis may be valuable in assessing possible transmission after MDA has ceased in order to determine re – emergence, especially in hyper-endemic communities. Defining endpoints for trachoma infection status may re-evaluate and steer trachoma elimination programs, and also allow for the integration of elimination and control programs of multiple NTDs.

Manuscript Tables and Figures

Figure 2. A map of the trachoma prevalence by district in Tanzania. Credit: Imperial College London, 2012.

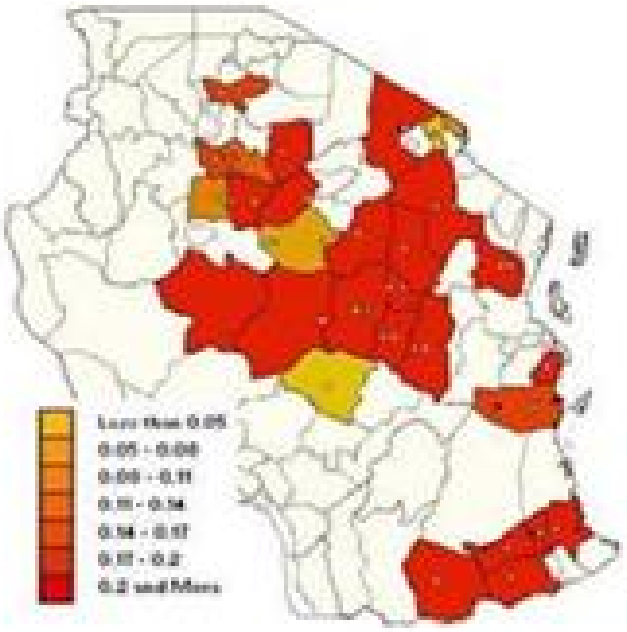


Figure 3. Median responses are shown in median florescence intensity minus background (MFI – BG) by country for A)pgp3 and B)CT694.

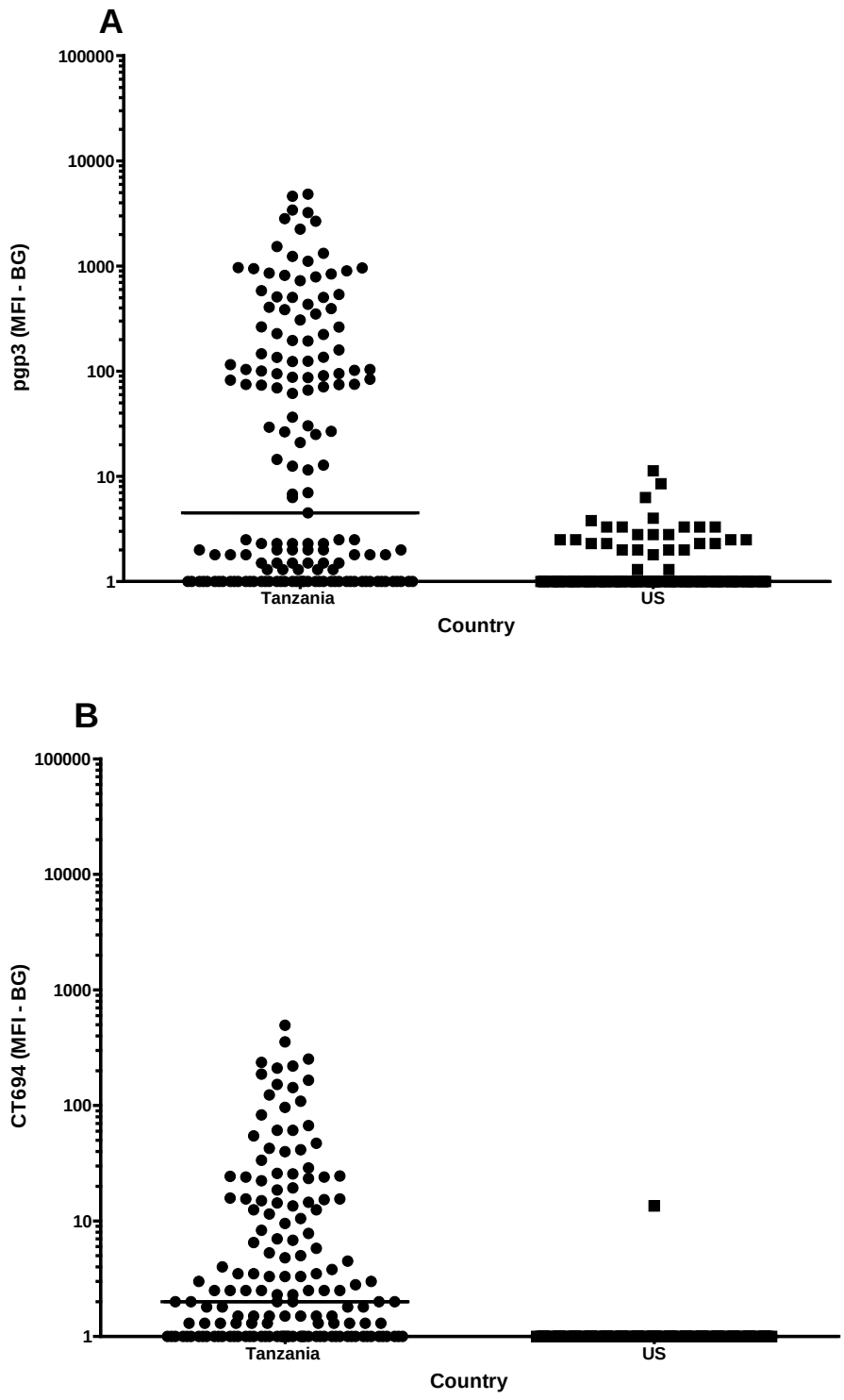
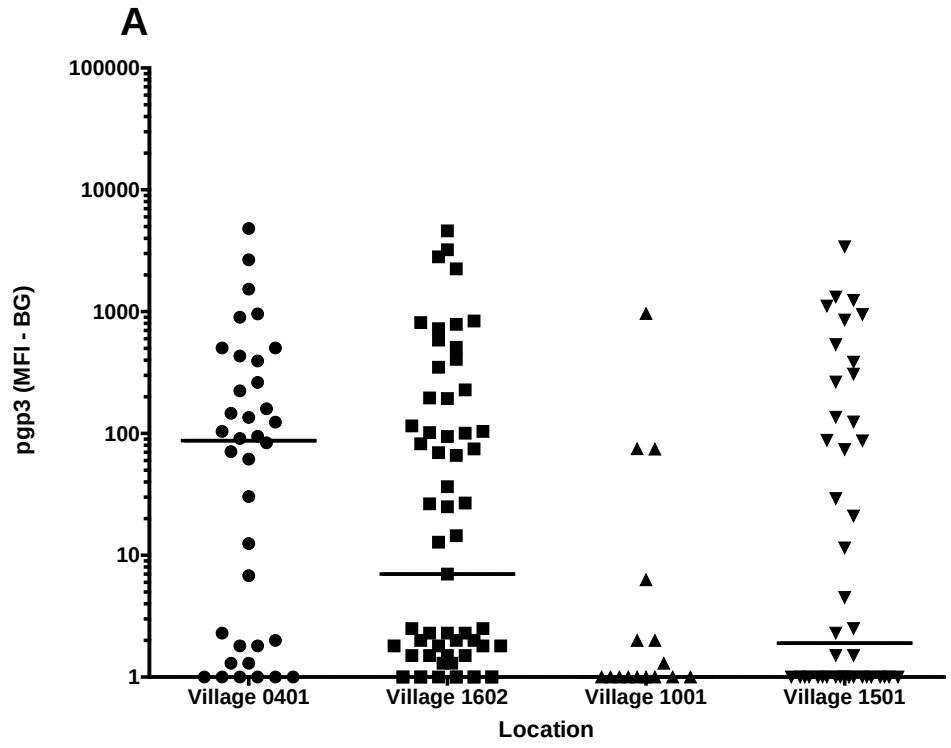


Figure 4. Median responses are shown in median florescence intensity minus background (MFI – BG) by Tanzanian villages for A) pgp3 and B) CT694.



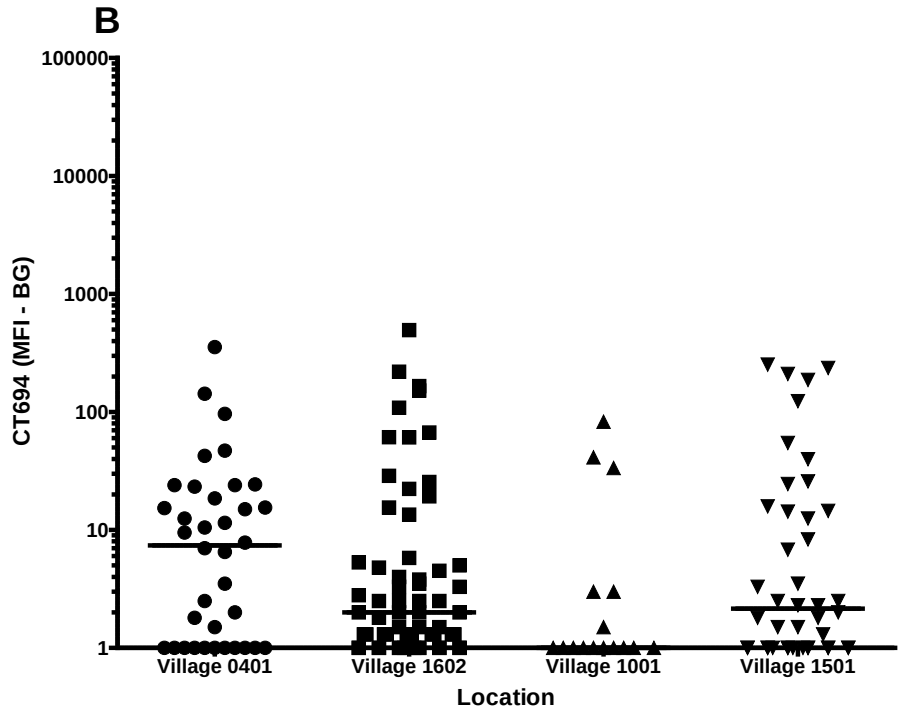


Figure 5. Median responses are shown in median florescence intensity minus background (MFI – BG) by clinical trachoma score for A) pgp3 and B) CT694. PCR positive children are indicated in red.

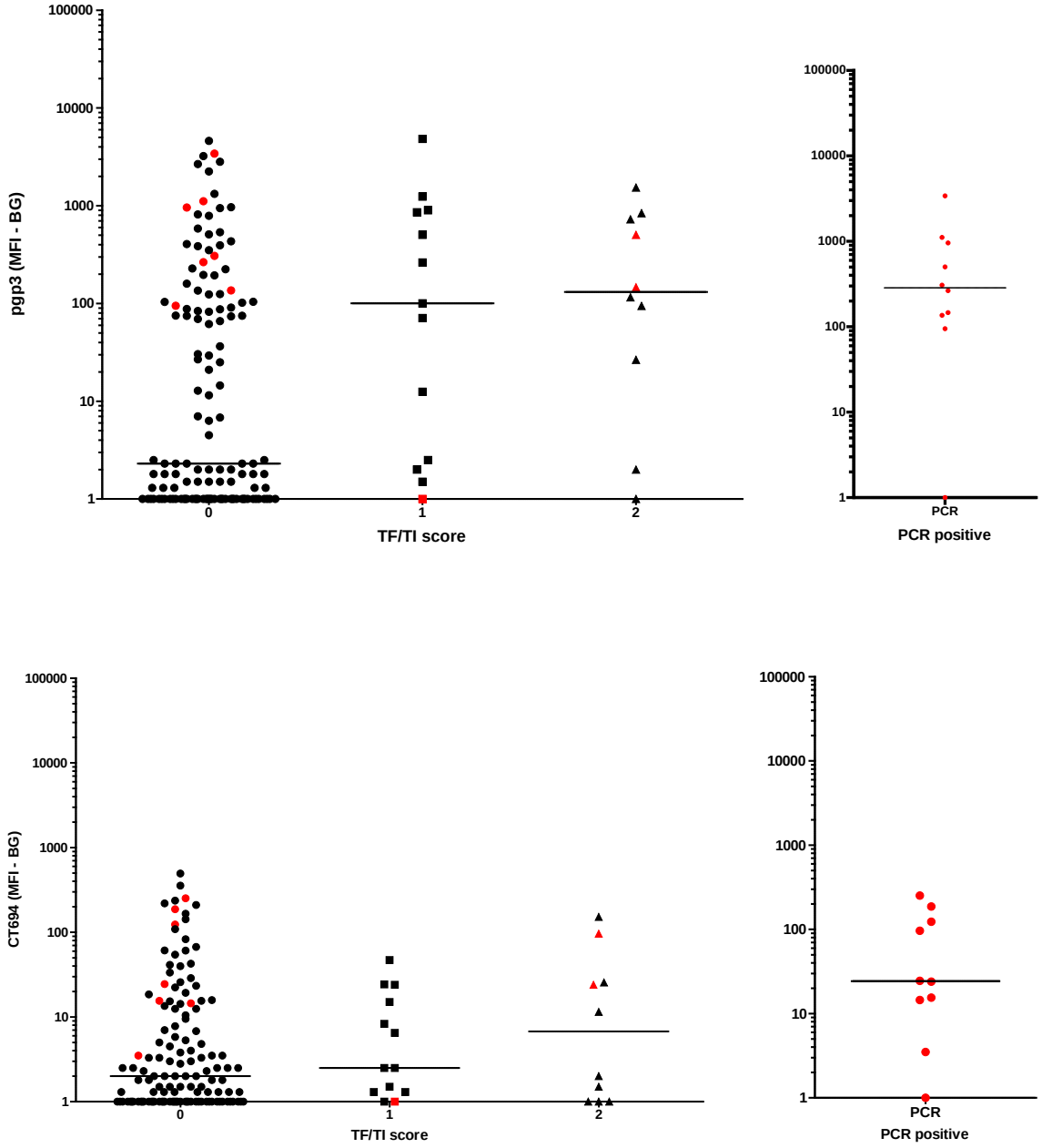


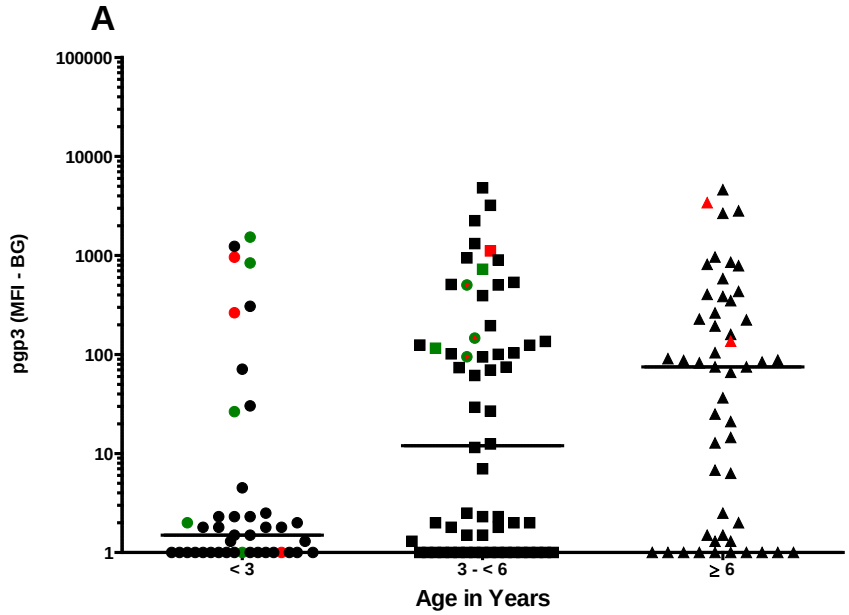
Table 2A. Distribution of ocular pathology (TF/TI = 1 or 2) and infection status for pgp3.

pgp3	**PCR +**	**PCR -**
TF/TI+	2/3	15/20
TF/TI-	7/7	50/125

Table 2B. Distribution of ocular pathology (TF/TI = 1 or 2) and infection status for CT694.

CT694	**PCR +**	**PCR -**
TF/TI+	1/3	7/20
TF/TI -	6/7	31/125

Figure 6 Median responses are shown in median florescence intensity minus background (MFI – BG) by age groups for A)pgp3 and B)CT694. PCR positive children are indicated in red, and individuals with a TF/TI score of 2 are indicated in green.



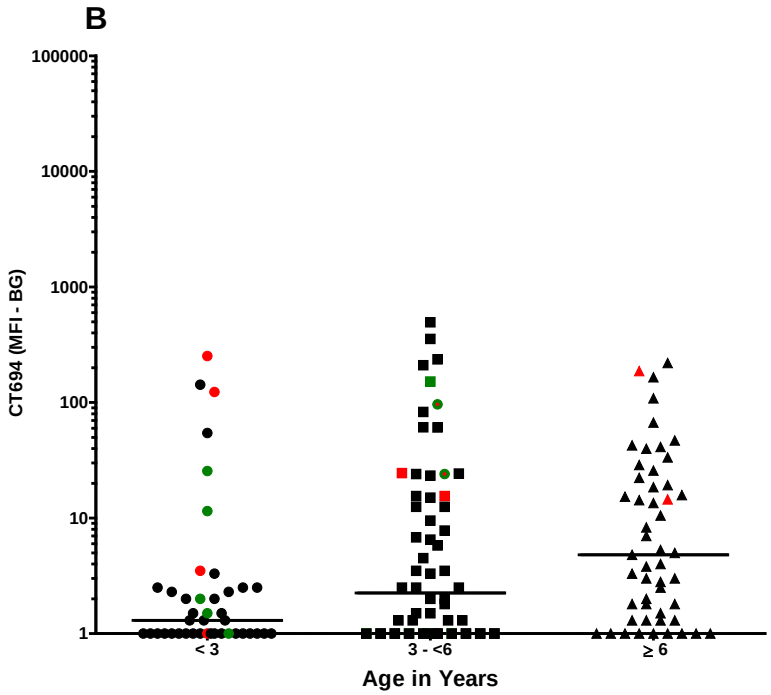


Figure 7. Median responses are shown in median florescence intensity minus background (MFI – BG) at baseline by clinical trachoma score for A) pgp3 and B) CT694. PCR positive children are indicated in red.

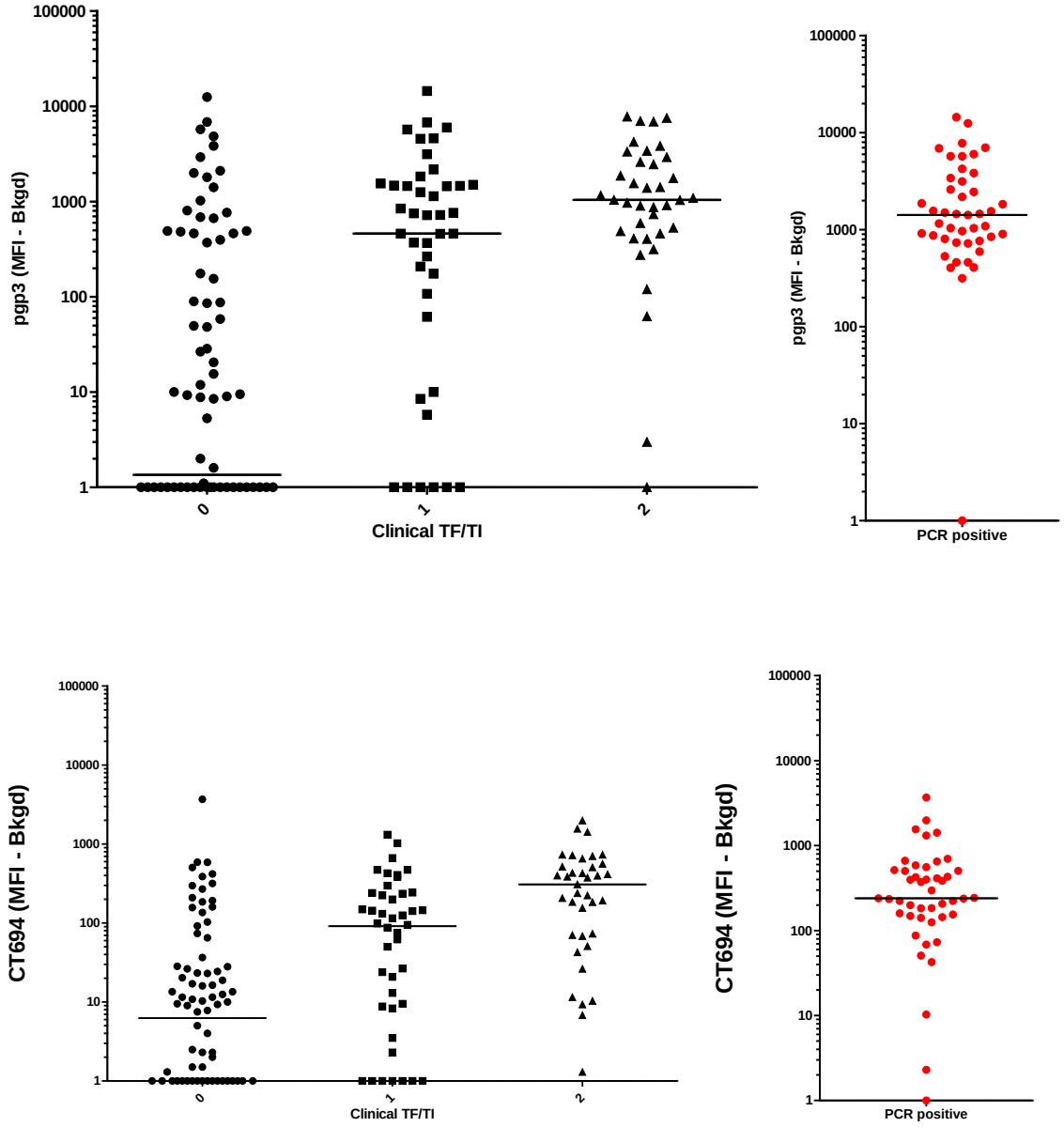
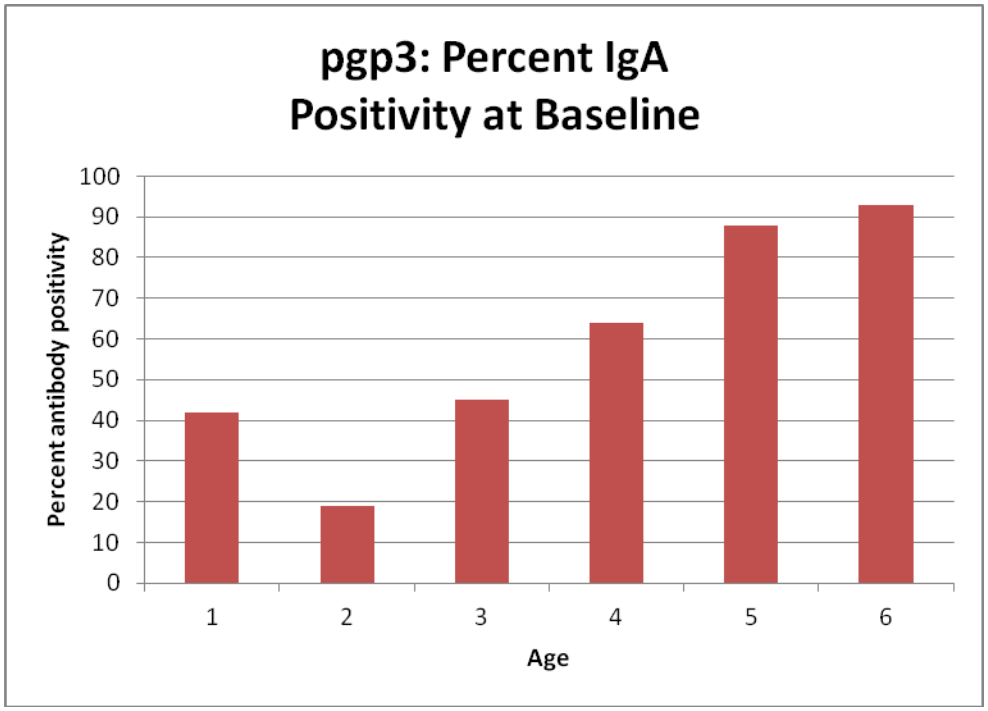


Figure 8. Percent IgA antibody positivity at baseline by age for pgp3 and CT694.



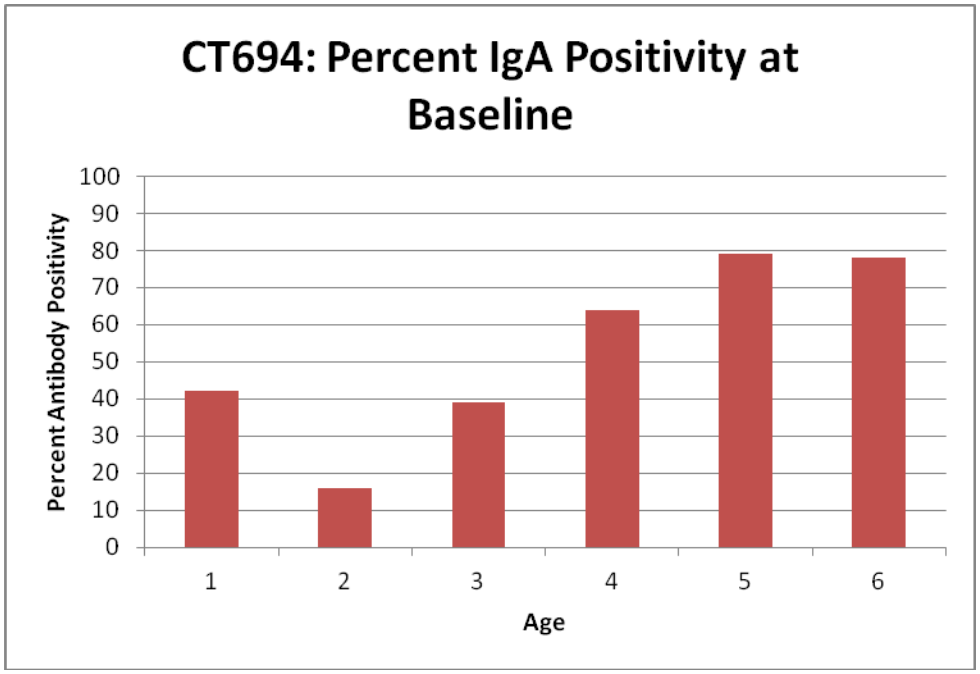
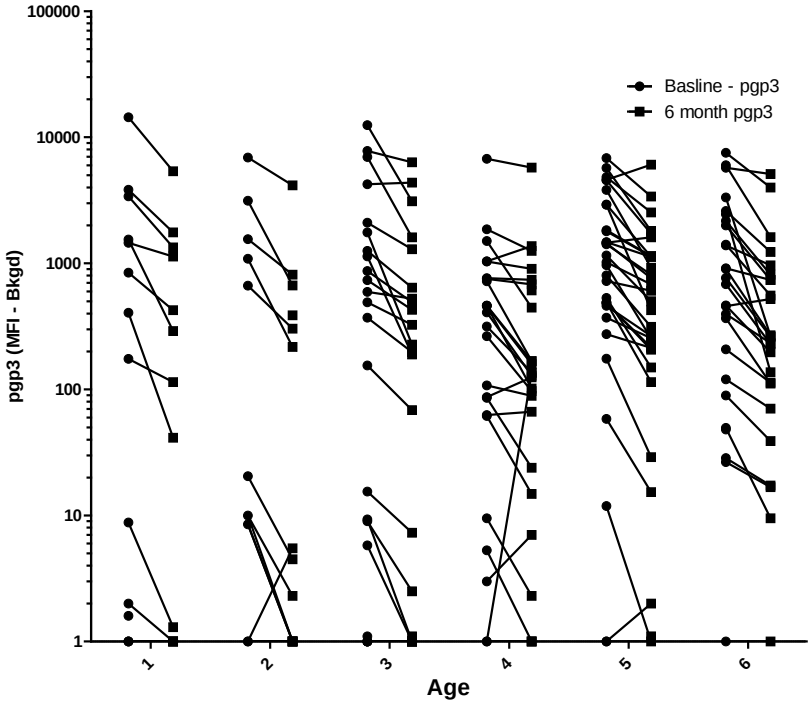
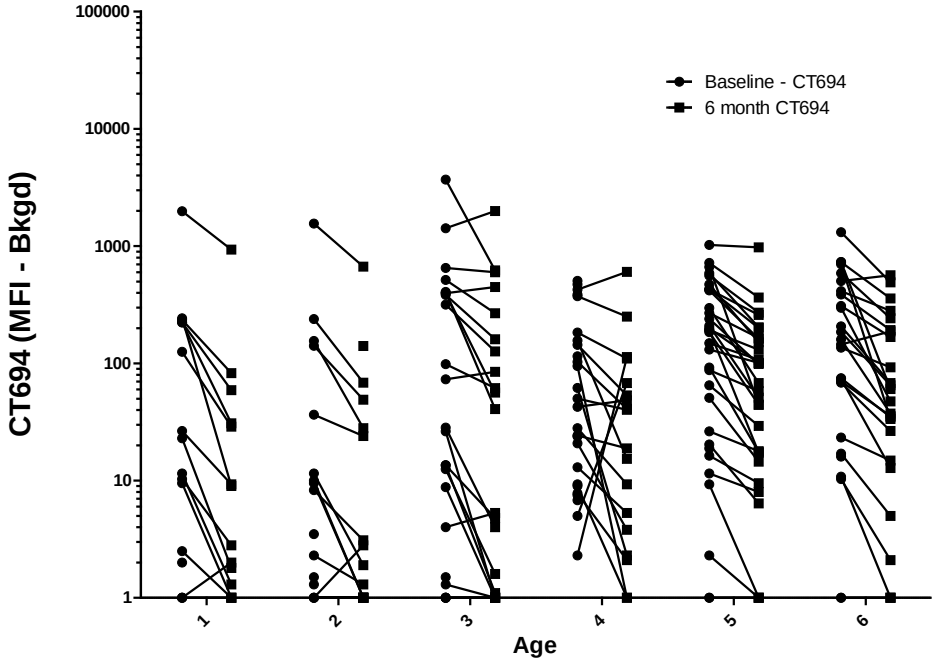


Table 3. Comparison of medians at baseline and six months post treatment stratified by age for each trachoma antigen, pgp3 and CT694. ** indicates a significant p-value, with alpha = 0.05.

Age	**Pgp3 Number (n)**	**CT694 Number (n)**	**Pgp3 p-value**	**CT694 p-value**
1	8	8	0.0078**	0.0078**
2	7	6	0.0781	0.2188
3	15	13	0.0004**	0.0574
4	22	21	0.0194**	0.0261**
5	28	28	< 0.0001**	< 0.0001**
6	25	22	< 0.0001**	0.0004**
Total	105	98		

Figure 9. Rate of antibody response decline per individual from baseline to six months post treatment by age for A)pgp3 and B)CT694.





Chapter 4: Conclusion and Recommendations

Serological testing shows promise in clearly defining active trachoma to produce a more accurate picture of current infection status and for defining endpoints for trachoma infection [2]. More clearly defining trachoma with the use of antibody tests will allow for fewer false positives that are seen in using the clinical exam alone, and will also be more cost effective than PCR analysis. Past studies in neglected tropical diseases reinforce the possibility of using antibody – based tests for monitoring of elimination programs after mass drug administration has ceased, and suggest the capacity for antibody based tools to drive mass drug administration, elimination efforts, and public health policy [8,9,10].

Current WHO recommendations for trachoma elimination use TF as an indicator for treatment, yet clinical symptoms persist even though infection has been cleared. Also, weak associations have been found between TF clinical manifestation and the presence of *C. trachomatis* in low – prevalence settings [4]. This may lead to overestimation in trachoma baseline threshold prevalence, and may provide misleading information on the current status and progress of trachoma elimination efforts. Also, though clinical trachoma graders undergo training, there may be variation between graders. Therefore, a misdiagnosis of ocular pathology may occur due to variation in diagnoses among graders.

4.1 Implementation

The advantages of the multiplex bead assay have been described in previous studies when compared to data output produced by PCR and other antibody tests, such as ELISA. Quantifying a cut off that is measured by antibody response fluorescence intensity may prove to be a beneficial alternative to the clinical examination to determine trachoma prevalence. Determining standardized cutoffs for antibody positivity for trachoma antigens will allow public health

practitioners to monitor individuals that are truly infected with trachoma. Using an antibody based test will increase the sensitivity and specificity of baseline threshold trachoma prevalence surveys, and provide a more accurate assessment of infection. Currently, no azithromycin resistance has been documented against trachoma. However, treating individuals that are not truly infected will misallocate antibiotics, and may create a selective pressure environment where azithromycin – resistant trachoma may thrive. Also, emerging evidence suggests that MDA for trachoma control is associated with an increased risk of azithromycin – resistant *Streptococcus pneumoniae* in young children when observed six months after treatment. This emerging evidence, as well as future studies, must be assessed in future MDA trials and trachoma control programs [63]. Trachoma may not exhibit azithromycin resistance; however, it is necessary to monitor other diseases that may be exacerbated with the selective pressure of mass drug administration for trachoma elimination. It is important that MDA not perceived as harmful to the target population, and emerging, resistant diseases are identified and assessed in MDA trials.

Communities infected with multiple NTDs may benefit from multiplex bead assay analysis. Previous studies have shown the capacity of its use in communities burdened with multiple infections, such as lymphatic filariasis and schistosomiasis[8,10]. By examining multiple disease-specific antigens in one analysis, integrated NTD prevalence surveys can be performed simultaneously. This suggests that the multiplex bead assays may serve as a cost effective method to surveying disease prevalence for multiple NTDs, versus utilizing separate, disease-specific survey methods. This may encourage and foster integration for disease elimination and control programs, and provide a more cost effective and efficient means of collecting prevalence data within infected communities.

The multiplex bead assay may prove to be valuable in surveillance of trachoma, as well as other diseases. Monitoring trachoma prevalence is necessary after MDA has ceased in order to determine if there is re-emergence of the disease in the community. By determining possible re-emergence of trachoma after MDA has ceased, public health practitioners may modify their elimination strategies for the specific community. Implementation of facial cleanliness and environmental improvements may be incorporated into elimination campaigns where MDA alone is unsuccessful in eliminating trachoma infection. Incorporating the other aspects of SAFE is important, particularly in hyper-endemic communities where re-emergence is more likely to occur. Monitoring antibody prevalence over time in hyper-endemic communities will allow for modifications to MDA – only elimination campaigns to incorporate the hygiene aspects of SAFE to interrupt trachoma transmission. By incorporating hygiene efforts in MDA – only campaigns, it may be possible to eliminate trachoma with fewer rounds of treatment. Also, monitoring trachoma antibody prevalence over time through multiple rounds of MDA may provide a clearer portrayal of the kinetics of antibody responses in hyper-endemic communities.

4.2 Recommendations

In the context of this study, further investigation of pgp3 and CT694 are required to determine differences between antibody positive responses. Individuals that are IgA positive for pgp3 are not necessarily positive for CT694. Further studies of the antigens may be able to reveal the reason for these differences. Also, due to the limitations of the study, a larger study population would be needed in order to verify the kinetics of IgA responses in a longitudinal study. A larger sample size will provide more power to the study, and may verify conclusions found in this study.

Results are not readily understood by program managers, and the assays require more experimentation and research within a laboratory setting before they can be used by program managers in trachoma – endemic communities. Currently, the assays require further research before the results can be used for decision making of public health programs and interventions.

4.3 Conclusion

The multiplex bead assay demonstrates value in the field of antibody testing for NTD surveillance. The assay's capability for simultaneous disease prevalence screenings allows for the integration between disease – specific elimination programs, which can foster collaboration within regions with co-infections. Collaboration and integration is key for NTD elimination and control, and the multiplex bead assay may serve as a possible candidate assay to determine disease prevalence at baseline, and to monitor disease prevalence throughout the course of MDA campaigns. The progression and further research of serological tools, such as the multiplex bead assay, may provide public health practitioners the data to drive public health policy in terms of NTD surveillance and treatment, and to modify current elimination programs specific to the community at risk.

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