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The Effect of Antigen Polymorphisms on Serological Antibody Detection Assays Based Upon the

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The Effect of Antigen Polymorphisms on Serological Antibody Detection Assays Based Upon the *Onchocerca volvulus* 16kDa Diagnostic Antigen (Ov16)

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
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DEDICATION

I would like to give special thanks to my husband, James Miley, my mother and stepfather, Rosemary and Brian Lee, and my children. I will be forever grateful for the numerous sacrifices and countless words of encouragement that made this work possible. I would also like to dedicate this thesis in honor of my late father, V. Lee Watson, whose love and compassion lives on in me, forever giving me strength, courage, and passion to succeed wherever life's journey may take me.

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ABSTRACT

Onchocerca volvulus is a filarial parasite transmitted to humans by female *Simulium spp.* black flies. Infection with this parasite can cause blindness and severe skin disease among humans in Africa and the Americas. Enzyme-linked Immunosorbent Assay serological testing of OV-16 antigen is a diagnostic tool for determining effective elimination of the parasite. Programs typically rely on OV–16 ELISA to evaluate the progress towards interruption and/or elimination of disease by mass drug distribution of ivermectin and vector larvicidal control efforts. As elimination grows closer, monoclonal antibody positive controls for OV-16 ELISA become important to develop for *Onchocerca* testing due to the limited availability of pooled sera positive controls. Recent evaluation of laboratory designed OV-16 ELISA coating antigen by the Unnasch Lab (University of South Florida) showed that polymorphisms occurred which may alter the ability of the humanized monoclonal antibody to recognize the cognate antigen. With this development, it was important to evaluate these polymorphisms and isolate them for further testing against the standardized monoclonal antibody and positive sera to determine the effects antigenic polymorphisms could have on diagnostic testing. Upon evaluation, the polymorphisms did influence signaling when testing the monoclonal antibody. However, little effect on the recognition of the antigen was seen when different isoforms were evaluated against sera from *O. volvulus* infected individuals. Data suggest that the epitope recognized by the synthetically produced monoclonal antibody is not immuno-dominant in infected individuals.

CHAPTER ONE:

INTRODUCTION

Onchocerciasis, sometimes referred to as “River Blindness”, is one of the neglected tropical diseases (NTDs) affecting humankind and has been documented as one of the most common causes of preventable infectious blindness (WHO, 2016). However, Onchocerciasis is also one of the NTDs which has the potential for elimination through vector control measures and the efforts of mass distribution of microfilaricidal treatments by multiple health response alliances with the support of the World Health Organization (WHO) (WHO, 2016).

This debilitating disease is caused by a parasitic filarial nematode known as *Onchocerca volvulus* (Coffeng, et al., 2013). Although there are other *Onchocerca spp.* which have been identified to infect mammals, such as; *O. ochengi* in cattle, *O. lupi* in canines, and *O. cervipedis* in deer, *O. volvulus* appears to selectively target humans as its only host (Boatin & Amazigo, 2016). Onchocerciasis has the potential to manifest in either an ocular form of illness, lymphatic involvement, or display in the form of filarial dermatitis, and the severity of disease appears to be linked to repeated exposure to infective bites (Dobson, 2008).

Human infection of *O. volvulus* occurs from the bite of a previously infected blackfly, which upon inoculation the L3 larvae will complete their cycle to reproductive

adulthood in approximately 24 months within the human host (Boatin & Amazigo, 2016; WHO, 2016). Adult female *O. volvulus* form nodules within the body, usually near bony prominences, where they have the ability to reside for upwards of 14 years and produce first stage larvae (microfilariae) at a reproductive potential of "700-1500 per day" (Liu, 2013). The microfilariae that are produced leave these nodules and take up residence in the skin, and in some cases the eye, where they can survive for up to two years (Liu, 2013). In this human infective stage, the microfilariae are taken up by the black fly vector during blood-feeding and perpetuate the cycle upon molting to L3 larvae within the black fly's thoracic flight muscles and eventually exit the labium during subsequent feeding to further transmission (McClelland, 1992).

It is estimated that 37 million individuals are infected with *O. volvulus* (Heymann, 2008). The disease predominantly affects individuals in sub-Saharan Africa wherein approximately 99% of onchocerciasis cases in the world occur, with a few foci also noted in the Americas and Eastern Mediterranean (Noma et al., 2014; WHO, 2016). There does appear to be a geographical component to the distribution and symptomology of the disease. There are noted divisions in distribution of disease where "blinding" illnesses appear to be more prevalent in the savannah foci, "non-blinding" illnesses tend to occur in forest foci and this might be explained by varying vector-parasite complexes with the different strains of O. volvulus (Boatin & Amazigo, 2016).

The black fly (*Simulium spp.*) vector for onchocerciasis prefers a habitat of fast flowing water found in rivers and streams. Unfortunately for developing countries, this has created a cyclical paradigm between onchocerciasis and malnutrition, as communities attempt to avoid the risk of disease they also regress to inferior farmland

(Dobson, 2008). Unfortunately, the plight of this disease does not stop with the illnesses that it evokes, it also causes economic strain on the affected communities. However, great efforts have been made in targeting these black fly environments with control measures as noted in early achievements of the Onchocerciasis Control Programme in West Africa (OCP), which initially started targeting habitat control with larvicides in the 1970's and later added human microfilaricidal treatments to their arsenal using ivermectin in the late 1980's (Dobson, 2008; Unnasch, 2004). Other programs such as; the African Program for Onchocerciasis (APOC) and Onchocerciasis Elimination Program in the Americas (OEPA) have mass treatment strategies in pursuit of interruption and eradication of *O. volvulus* (Eisenbarth et al., 2016).

With control program measures in place and ongoing human treatment with mass drug administration (MDA) of "Mectizan ® from Merck & Co.", onchocerciasis is making its way towards elimination (Schwab, 2007). In fact, recent research indicates that Colombia, Ecuador, Mexico, and Guatemala have reached elimination status, and several foci have thought to have interrupted the disease, including foci in Uganda and the Sudan (Boatin & Amazigo, 2016; Higazi et al., 2013; WHO, 2016). As research continues to evaluate the progress of the MDA program and vector control measures it appears that onchocerciasis elimination is possible within endemic regions still affected by disease. That being said, as interruption and elimination approaches, challenges will inevitably continue to arise in determining the future risk of reemergence in previously endemic regions.

The gold standard of diagnosis for *O. volvulus* has historically been through the microscopic evaluation of skin biopsies (skin snips) for the appearance of microfilariae

after clinical pathology of disease has been determined via ocular damage, palpable nodules, or perhaps by dermatological symptoms (Liu, 2013). Generally, these skin snips are obtained using a sclerocorneal biopsy punch that excises approximately 2.5mm of tissue, that is then incubated in culture media or saline wherein the microfilariae exit the tissue and the fluid can be examined for their presence (Boatin & Amazigo, 2016; Liu, 2013). Unfortunately, this diagnostic method can be painful and could potentially result in underestimation of disease burden due to limitations in the number of accommodating participants.

The paradox of MDA treatment programs is that it may lead to fewer detectable microfilariae in each patient and could allow for underestimation of disease in foci that are near or at elimination status. Assays like the Ov-16 enzyme-linked immunosorbent assay (ELISA) are used to geographically map the distribution of onchocerciasis and monitor control program progress, but many such filariasis assay applications have been noted to have issues regarding cross reactivity and deficiencies with standardization (Weil, et al., 2011). Currently, the Ov-16 ELISA is used to evaluate successful suppression of the disease by investigating potential exposure to the parasite in children less than 10 years old (Cupp, et al., 2012). Exposure is determined by the ELISA detecting IgG4 antibodies against Ov16, a 16kDa immunodominant antigen. Children are tested as sentinels for continued or emerging exposure, as Ov-16 ELISA is limited to evaluating antibodies that are exhibited post exposure which may also be present in individuals who were previously infected and treated.

As elimination approaches in a given region, the limited availability of positive sera for use as controls in diagnostic tests like Ov-16 ELISA adds to the complex

challenges of having sufficient diagnostic tools available for monitoring the progress towards onchocerciasis elimination (Golden et al., 2016). Recently, a humanized monoclonal antibody was produced as a positive control for use in Ov-16 ELISA to help solve this dilemma (Golden et al., 2016). As low levels of parasitemia become more common due to the current MDA programs, the monoclonal antibody design seems an advantageous alternative to the previous techniques of pooled sera. Designing standardized positive controls also reflects the need to have universal applications and reproducibility of these required conjugates in the current onchocerciasis surveillance programs in order to evaluate various foci and potential elimination and/or transmission status properly.

Upon evaluating the efficacy of the humanized monoclonal antibody against Ov-16 ELISA within the Unnasch laboratory (University of South Florida), difficulties arose in the ability of the Ov-16 antigen to appropriately detect the humanized monoclonal antibody. It was determined that this monoclonal antibody, produced as a positive control for the Ov-16 ELISA, reacted to a recombinant version of the Ov-16 antigen derived from parasites from Guatemala and was unable to bind to a homologue derived from a sequence obtained from parasites from Cameroon. It is not uncommon to exploit the antigen antibody interactions in designing surveillance test formats when diagnosing filarial infections, but there are known drawbacks including cross reactivity with nematode infections other than the target, in this case *O. volvulus* (Lammie, 2004). Therefore, designing a humanized monoclonal antibody that recognizes the immunodominant antigen appropriately with specificity for an *Onchocerca* testing platform is important.

Through investigation, the Unnasch laboratory discovered that polymorphisms occurring in the Ov-16 coating antigen may further explain the effect on the ability of the monoclonal antibody to recognize the antigen via Ov-16 ELISA assay. Upon genetic analysis by the Unnasch laboratory, it was discovered that two distinct polymorphisms had occurred at amino acids 167 & 196 in the Ov-16 antigens derived from Guatemala and Cameroon. The polymorphisms found differing in the Cameroon antigen had altered at amino-acid 167 arginine to proline (167R>P) and 196 arginine to proline (196R>P) (Fig. 1).

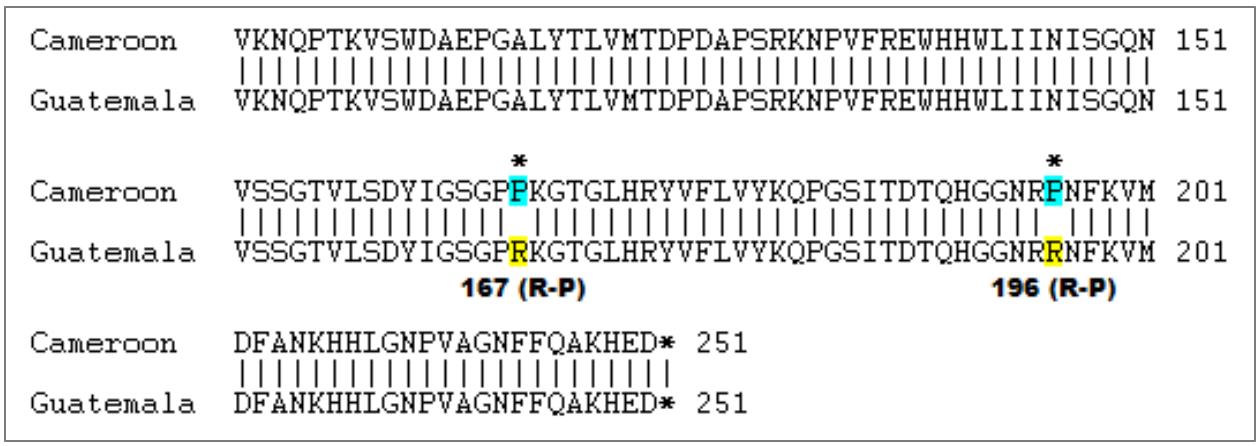


Figure 1: Amino acid sequence of *O. volvulus* parasites showing polymorphisms

Having determined these polymorphisms were present, it was important to evaluate them in the Ov-16 coating antigen and isolate them for further testing. Clones were produced by *in-vitro* mutagenesis for each of the antigens to be tested; one clone

containing no mutations reflecting the Guatemalan strain (parental construct), one clone containing a single mutation at 167R-P (arginine to proline), one clone containing a single mutation at 196R-P (arginine to proline), and one clone containing both mutations. Each of the mutated Ov-16 antigens was expressed with a GST tag and purified using affinity chromatography. Once purified, these four laboratory designed Ov-16 antigens (OV-16:167R196R, OV-16:167P196P, OV-16:167P196R, & OV-16:167R196P) could then be tested against the monoclonal antibody, as well as a set of known positive sera via Ov-16 ELISA in order to identify the polymorphism responsible for the loss in monoclonal activity and to determine if the polymorphisms affected the sensitivity of the Ov-16 ELISA.

The aim of this research was designed to investigate the performance of Ov-16 ELISA with respect to four variant recombinant Ov-16 antigens to evaluate the noted polymorphisms. Objective comparisons of these Ov-16 antigens containing isolated polymorphisms to an Ov-16 antigen that contained no polymorphisms were performed to determine whether or not these isoforms infer functional similarity on serological testing within the Ov-16 ELISA platform. Investigation regarding reactivity to the humanized monoclonal antibody was also performed to determine the effects antigenic polymorphisms may have on such synthetic positive controls. With onchocerciasis interruption and elimination in sight and fewer infections occurring, future test applications may soon rely on the development of adequate humanized monoclonal antibody standardization techniques. However, these synthetic positive controls should be designed to react appropriately with an immunodominant antigen that is specific to *O. volvulus* and sensitive to detection when present in sera if it is to replace the need for

pooled sera positive controls in current onchocerciasis surveillance programs (Lucius, 1988). Furthermore, the World Health Organization's certification of elimination protocol relies heavily on the Ov-16 ELISA testing platform in their Onchocerciasis surveillance programs which warrants quality control efforts to ensure that these serological assays perform to the highest standards possible, as the results of such testing will ultimately dictate how screening children in each focus will proceed (Cupp, et al., 2012).

CHAPTER TWO:

MATERIALS AND METHODS

Isolation and Purification of Mutations for Use in OV-16 ELISA

Four clones were produced, one for each of the laboratory Ov-16 antigens, by *in-vitro* mutagenesis and plasmids (pGEX, a commonly used expression vector) were maintained at -80^{o} C, by Dr. Canhui Liu in the Unnasch laboratory. 1ul of each of the plasmid was inoculated into 25ul BL-21 competent *E-coli* cells and incubated on ice for 5 minutes then heat shocked at 42^{o} C 45 seconds. They were immediately placed on ice for two minutes after which time 900ul of SOC buffer was added and tubes were incubated in platform shaker at 37^{o} C for one hour. After incubation, a 1:10 dilution of positive cells was created and 125ul of cells were plated on LB Agar plates containing Ampicillin at a concentration of 100ug/ml, along with negative control plates, and incubated upside down at 37^{o} C overnight. Positive plates were examined the next day for presence of colonies, as well as no colonies confirmed in negative control plates. Individual *E. coli* colonies containing the transformed plasmids were chosen from each of the Ov-16 plasmid clones and grown using LB media containing Ampicillin100ug/ml in step-wise proportions starting with 5ml of media incubated overnight at 37^{o} C and shaking at 300 RPM. The 5ml overnight culture was then diluted to 100ml with LB media containing Ampicillin 100ug/ml and incubated overnight under the same

conditions. 50ml of this overnight culture was then added to 1 liter of LB media containing Ampicillin 100ug/ml, incubated at 37^{o} C and 300 RPM to mid-log phase by monitoring optical densities at A_{600} to a value between 0.6 - 1.0. Once these values were reached, protein expression was induced by adding isopropyl-β D-thiogalactoside (IPTG) to a final concentration of 0.1mM. An additional OD reading was taken 1 hour following the addition of IPTG to confirm growth was continuing and the cells were allowed to grow overnight at 37^{o} C and 300 RPM. After growth was complete, the media containing each protein/Ov-16 antigen was then divided into 250ml cell centrifuge tubes and the cell pellet concentrated via centrifugation at 13,000 RPM for 10 minutes at 4^{o} C. Cells are resuspended in cold GST binding buffer (150mM NaCl, 25mMTris, 1mM EDTA) containing protease inhibitors (Thermo ® Protease Inhibitor tablets Cat.#78430) and 100ug/ml lysozyme. This was then followed by the addition of Triton x100 detergent to a final concentration of 0.5% to assist in lysing cells after which the cell pellets were placed at -80^{o} C overnight.

Each of the isolated Ov-16 antigens was individually purified by affinity chromatography using GSTrap HP® columns (GE Healthcare Life Sciences). This process began by thawing the cell pellets and sonicating them at 15% for 10 seconds with a one minute pause on ice, over three intervals to lower the viscosity. The cell debris was then centrifuged at 18,000rpm for 30 minutes at 4^{o} C. and the supernatant was retained and filtered through a 0.45um filter prior to its application to the affinity column. Using a separate 1.0ml GSTrap® column for each of the four Ov-16 antigens, all were purified under the same conditions using an infusion pump at a wash flow-rate of 1.0ml/min., a sample loading flow-rate of 0.2ml/min., and an elution flow-rate of

1.0ml/min. wherein all samples and buffers were maintained on ice. Important to note that the slower flow-rate for sample loading is recommended for increased binding of desired protein. The following buffers were used in all four purifications; binding buffer – 10mM sodium phosphate,140mM NaCl, 2.7mM KCl at pH of 7.4 and elution buffer – 50mM Tris/HCl, 10mM reduced glutathione at a pH of 8.0. The elutions retrieved from the four purifications were evaluated via the Nano-drop® and SDS gel electrophoresis to confirm presence of desired Ov-16 protein prior to following up with dialysis on each of them. Dialysis was performed on each protein using 3.0ml Thermo Scientific Slide-A-Lyzer ® dialysis cassettes immersed in 1X PBS. SDS gel electrophoresis was then repeated on the four Ov-16 antigens post dialysis. Each of the Ov-16 antigens was given a label designation based on their mutation characteristics and yield concentrations were calculated via Bradford ® Protein assay (Table 1).

Purified OV-16 protein ID	Yield Concentration in mg/ml	Polymorphism Characteristics
OV-16:167R196R	2.0 mg/ml	Parental construct
OV-16:167P196P	2.0 mg/ml	Double mutation; 167R>P & 196R>P
OV-16:167P196R	0.5 mg/ml	Isolated single mutation; 167R>P
OV-16:167P196R	1.0 mg/ml	Isolated single mutation; 196R>P

Table 1: List of purified OV-16 proteins with yield concentrations in mg/ml

Monoclonal Standards via OV-16 ELISA Against 4 Experimental Antigens

In previous research performed by Golden et al. (2016), a humanized monoclonal antibody was designed for positive controls in the OV-16 ELISA. Using a

human combinatorial antibody library (HuCAL & HuCAL Platinum), they were able to identify 15 unique antibody clones with specificity for Ov-16, which were then narrowed down to two based on binding affinity in ELISA and nitrocellulose platforms (Golden et al.,2016). Ultimately one recombinant antibody clone was chosen for development (AbD19432_hIgG4) based on absorbance range and signal strength (Golden et al.,2016). As previously noted upon testing in the Unnasch Laboratory, it was found that this humanized monoclonal antibody produced as a positive control for the Ov16 ELISA reacted to a recombinant version of the Ov16 antigen derived from parasites from Guatemala but did not react to a homologue derived from a sequence obtained from parasites from Cameroon. After careful isolation of the polymorphisms, the experimental Ov-16 mutations were tested individually against the humanized monoclonal antibody (AbD19432_hIgG4). The monoclonal antibody was serially diluted and tested via OV-16 ELISA techniques using the four experimental OV-16 antigens to evaluate detection. Optical densities were evaluated via spectroscopy at 405nm as per OV-16 ELISA protocols and results of each of the four antigens were compared.

Testing Ov-16 Polymorphisms in Sera via ELISA

To determine the effects that Ov-16 antigenic polymorphisms may have on serum reactivity regarding naturally acquired human antibodies in endemic regions, the four recombinant Ov-16 antigens were evaluated via OV-16 ELISA against 704 specimens that were collected from Liberia and Ghana. These serum samples contained no personal identifiers and the University of South Florida's IRB ruled on

9/16/16 that this work did not meet the definition of human subjects research. Standard Ov-16 ELISA protocols were used to evaluate the sensitivity of the four experimental Ov-16 antigens against the 704 serum samples. All 704 samples were previously determined positives for *O. volvulus* via skin snip results. For effective comparison of serum reactivity, each of the ELISA 96-well microtiter plates were divided such to be coated with all four experimental Ov-16 antigens (100ul per well) at 2.0ug/ml in carbonate buffer ($NaHCO_3$) (Fig. 2).

	1	2	3	4	5	6	7	8	9	10	11	12
A	McAb 128 MU	McAb 128 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
B	McAb 64 MU	McAb 64 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
C	McAb 32 MU	McAb 32 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
D	McAb 16 MU	McAb 16 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
E	McAb 8 MU	McAb 8 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
F	McAb 4 MU	McAb 4 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
G	McAb 2 MU	McAb 2 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control
H	McAb 0 MU	McAb 0 MU	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	PBST-BSA - Control

Coating Antigens	
OV-16:167R196R	(NO MUTATIONS)
OV-16:167P196P	(BOTH MUTATIONS 167R>P,196R>P)
OV-16:167P196R	(SINGLE MUTATION 167R>P)
OV-16:167R196P	(SINGLE MUTATION 196R>P)

Figure 2: OV-16 ELISA; Plate Map of 4 recombinant antigens

Samples were then tested at 1:80 dilution in 1X PBST/5% BSA (Phosphate buffered saline, 0.05% Tween20/ Bovine Serum Albumin) and 1ul of the humanized monoclonal antibody was diluted in 499ul of 1X PBST/5% FBS (Phosphate buffered saline, 0.05% Tween20/ Fetal Bovine Serum) which was then serially diluted using 1X PBST/5% FBS. All samples were tested in duplicate with each of the experimental Ov-16 antigens under the following conditions; plates were coated with 100ul at a concentration of 2ug/ml of each experimental antigen; Ov-16:167R196R, Ov-16:167P196P, Ov-16:167P196R, and Ov-16:167R196P as shown in figure 2 and incubated at 4^{o} C overnight. After incubation, the plates were washed four times with 1X PBST and dried post fourth wash. All plates were then blocked with 1X PBST/5%BSA and incubated for one hour at 4^{o} C. During this incubation step samples were diluted 1:80 and monoclonal antibody standards were diluted. After blocking step, the plates were emptied and dried without washing and sample standards and controls were added to wells and incubated at room temperature for two hours. After incubation, the plates were washed four times with 1X PBST, but dried after both the first and last washes. An anti-human (Mouse) IgG4 antibody conjugated to biotin was then added to all plates at a dilution of 1:1000 in 1X PBST and incubated at room temperature for one hour. 1XPBST washes were repeated four times and plates were dried. Streptavidin-AP (Streptavidin, Alkaline Phosphatase), a conjugate used to detect biotin in signal amplification in combination with chromogenic or fluorogenic substrates, is added to the plates in a 1:2000 dilution in 1X PBST and incubated at room temperature for one hour. Washes were repeated four times, plates were dried, and PNPP (p-Nitrophenyl phosphate) 1mg/ml solution was added to the plate wells. Plate optical densities were

evaluated at 405nm using a BioTek® microplate reader to optimum signal output upon which the plates exposure was stopped using 3M NaOH. Results were analyzed for sensitivity and the test efficacy of the non-mutated Ov-16 antigen was compared between each of the mutated Ov-16 antigens.

Testing GST Cross-Reactivity in Sera via Ov-16 ELISA

Plates were coated with GST at 2.0ug/ml in carbonate buffer, with the exception of four wells that were coated with Ov-16:167R196R (parental construct) at 2.0ug/ml in carbonate buffer for use with positive and negative controls. The plates were incubated overnight at 4^{o} C and the protocols were followed the same as during testing Ov-16 polymorphisms in sera via ELISA. The serum samples were tested in duplicate, with two negative controls and two positive controls per plate. Results were evaluated for signal at 405nm and any presence of cross reactivity.

CHAPTER THREE:

RESULTS

Analysis of Mutations Against the Monoclonal Antibody

Upon evaluation of the Ov-16 ELISA that was performed using a set of pooled sera and the monoclonal antibody standards, it confirmed that the antigen containing the polymorphisms at 167 (R-P) & 196 (R-P) was unable to detect the monoclonal antibody appropriately (Fig. 3), while the parental antigen detected the monoclonal

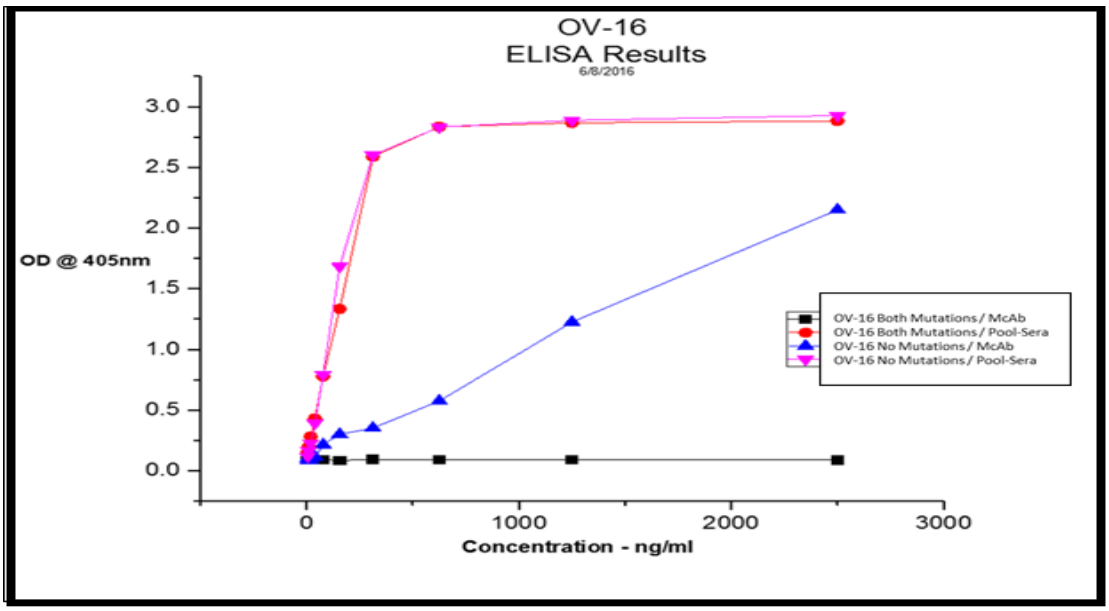


Figure 3: OV-16 ELISA; testing efficacy of monoclonal antibody

antibody signal as expected (Fig.3). Taking this information into account led to isolating the polymorphisms so that they could be evaluated further against the monoclonal antibody.

Isolated Mutations for Use in OV-16 ELISA

After purification of the four experimental Ov-16 antigens was completed via GSTrap HP® and Dialysis, an SDS gel electrophoresis was performed to identify the presence of the Ov-16 protein. Ov-16, a 16 kDa protein, which appeared at approximately 42 kDa when tagged to GST which has a value of 26 kDa (Fig. 4).

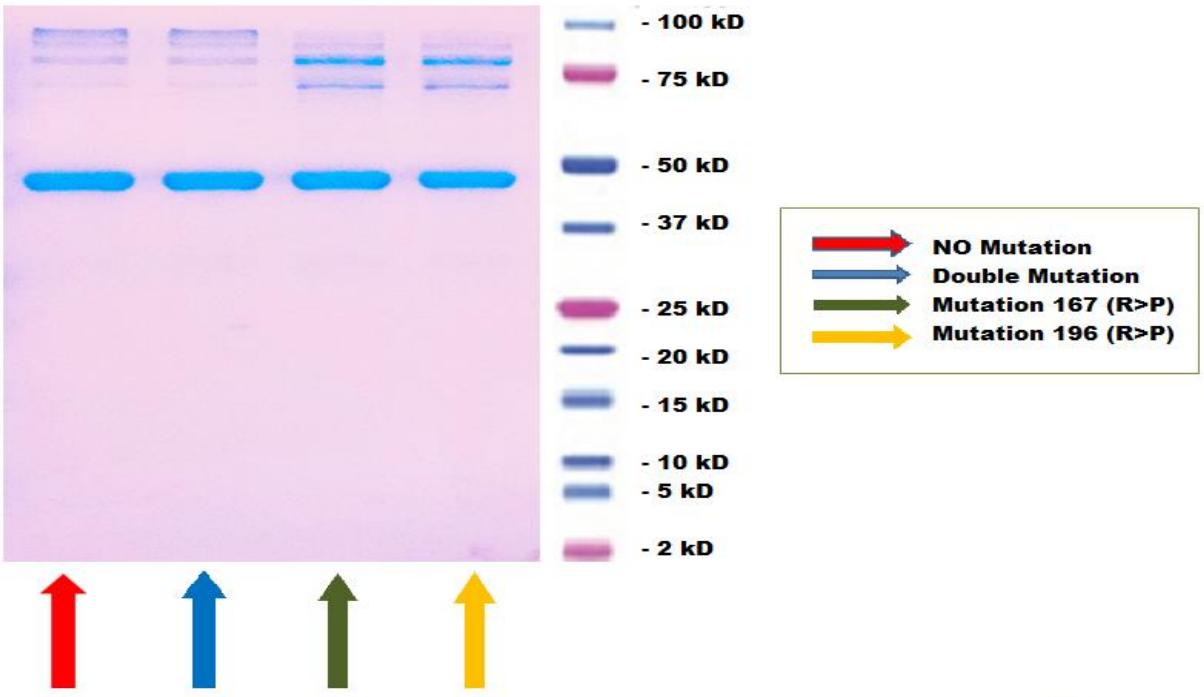


Figure 4: SDS Analysis of purified OV-16 proteins

The SDS gel confirmed the presence of the desired Ov-16 protein in each of the four experimental antigens that were required to move forward with testing each of them against the monoclonal antibody and the 704 positive sera.

Monoclonal Reactivity to Four Recombinant Antigens

The Ov-16 antigen parental construct (Ov-16:167R196R) and Ov-16 antigen containing a single mutation at 196 R>P (Ov-16:167R196P) displayed an OD @ 405nm which gave results indicating equal and strong binding to the humanized monoclonal antibody via Ov-16 ELISA (Fig. 5 a & b). In contrast, the Ov-16 antigen containing both mutations (Ov-16:167P196P) and Ov-16 antigen containing a single mutation at 167 R>P (Ov-16:167P196R) were not capable of binding the monoclonal antibody (Fig. 5 c & d).

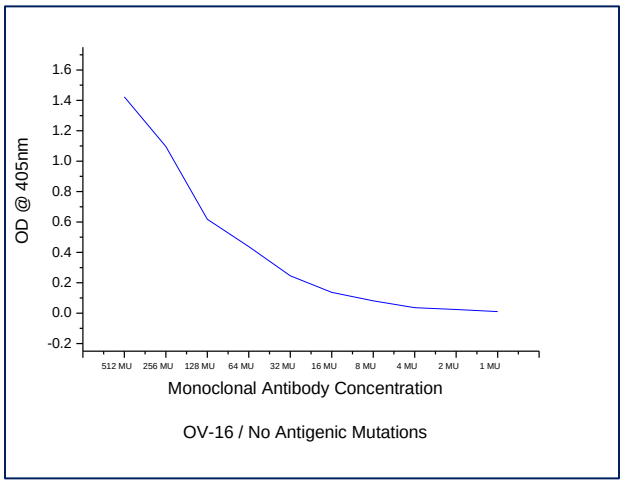


Fig. 5 a

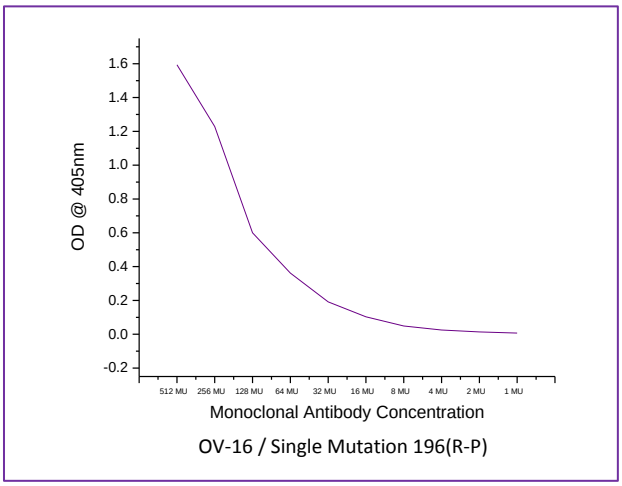


Fig. 5 b

Figure 5: Monoclonal reactivity to the four recombinant OV-16 antigens (a-d)

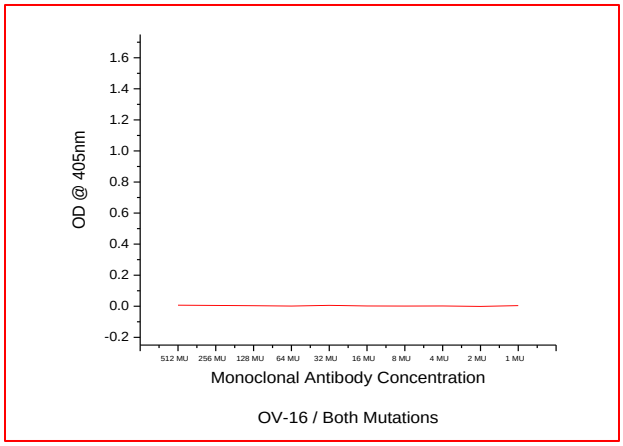


Fig. 5 c

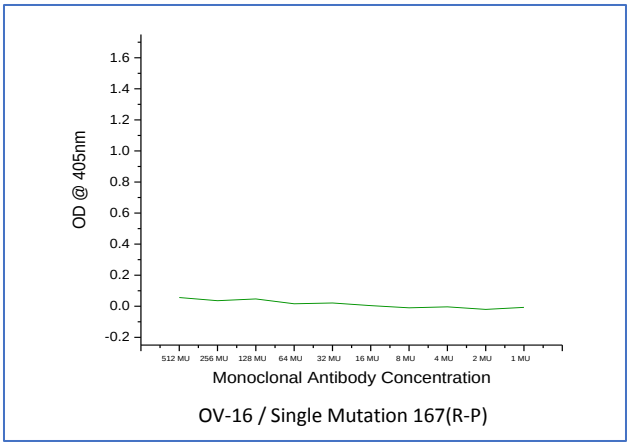


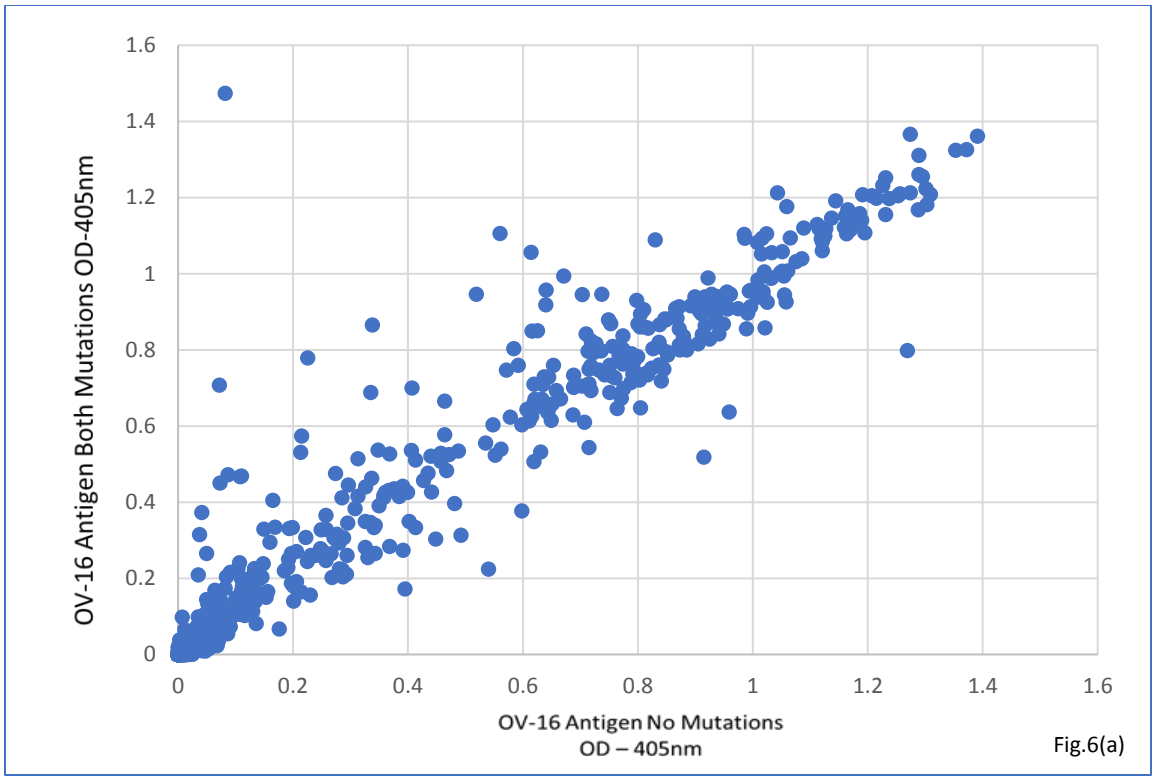
Fig. 5 d

Figure 5: Monoclonal reactivity to the four recombinant OV-16 antigens (a-d)(Cont.)

These data suggest that the mutation occurring at amino-acid 167R>P (arginine to proline) in the Ov-16 antigen disrupted the epitope recognized by the monoclonal antibody.

Serum Reactivity to Four Recombinant Antigens via Ov-16 ELISA

The positive control monoclonal antibody and patient serum samples (n=704) were tested via Ov-16 ELISA against the four recombinant antigens where patient sera were drawn from a serum bank collected in the 1980s in Liberia and Ghana; all individuals in this serum bank had positive skin snips, indicative of infection with *O. volvulus*. Results of the Ov-16 ELISA showed similar reactivity of the four experimental antigens with regards to patient sera, wherein the antigens which contained some form of mutation (Ov-16:167P196P, Ov-16:167P196R, & Ov-16:167R196P) were compared to the parental construct Ov-16 antigen (Ov-16:167R196R) (Fig. 6 a-c).



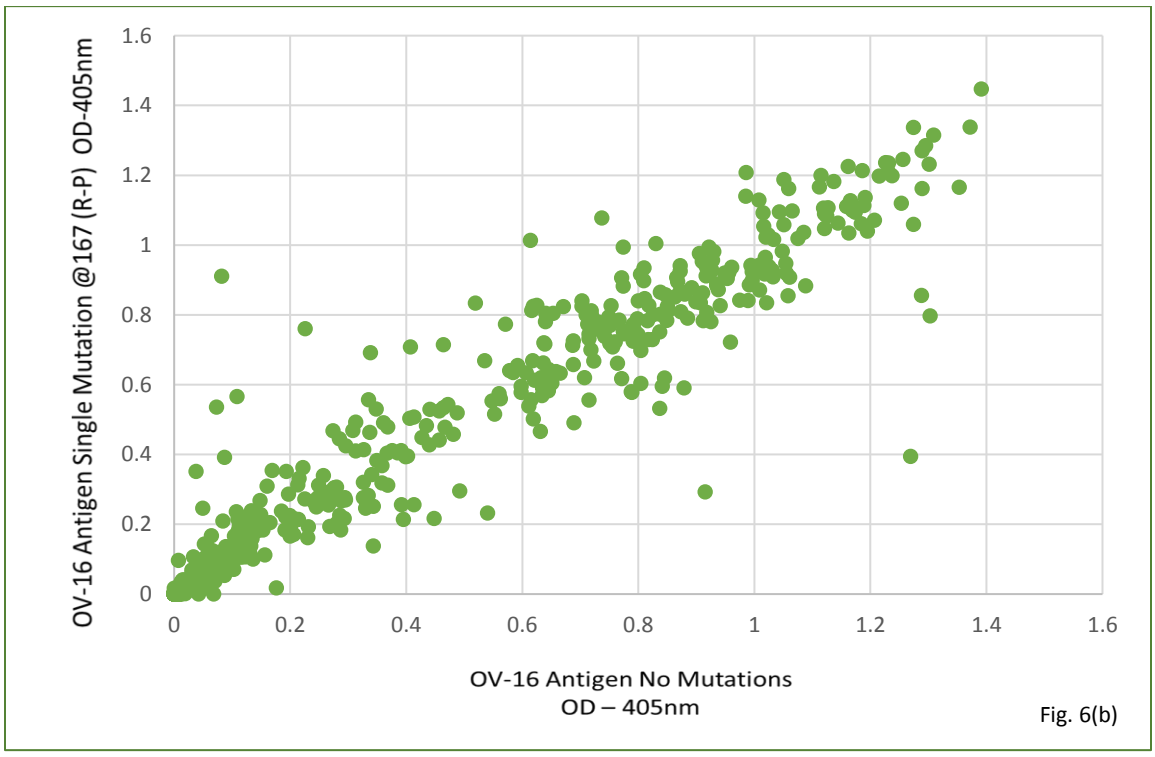


Figure 6: Comparison of serum reactivity to four recombinant antigens (a-c)

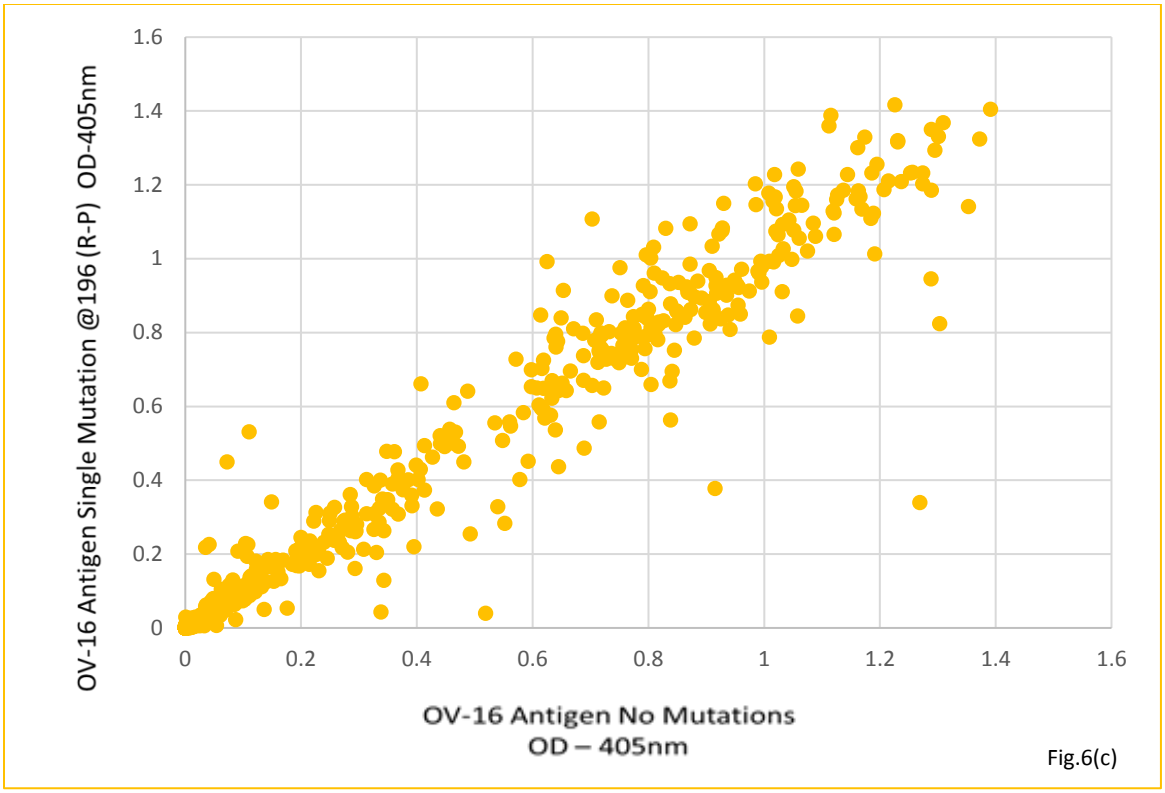


Figure 6: Comparison of serum reactivity to four recombinant antigens (a-c) (Cont.)

OV-16 ELISA Sensitivity of Polymorphisms

Upon evaluating whether a mutation had greater or lesser sensitivity than the parental construct Ov-16 antigen, it appeared that only a slight increase in sensitivity could be achieved when combining the antigen containing both mutations with parental construct Ov-16 antigen. Combining these Ov-16 antigens together increased sensitivity from 60.0% in Ov-16:167R196R (parental construct) to 63.5% by combining Ov-16:167R196R (parental construct) with Ov-16:167P196P (containing both mutations) (Fig. 7). Upon initial investigation, it was pondered that the combined efforts of these Ov-16 antigens could possibly increase the sensitivity of the ELISA, which could then be

initiated into current protocols to optimize testing in the field. After careful evaluation however, it appears that the combined antigen sensitivity does not imply statistical significance in this case. Statistical significance was evaluated using Chi-squared test. In this case, Ov-16:167R196R (parental construct) and the combined Ov-16:167R196R (parental construct) with Ov-16:167P196P (containing both mutations), both had a sample size n= 704 with percentages at 60.0% and 63.5% respectively. Based on Chi-squared test the P value was 0.18 indicating that no statistical significance exists for increased sensitivity when the Ov-16 antigens are combined.

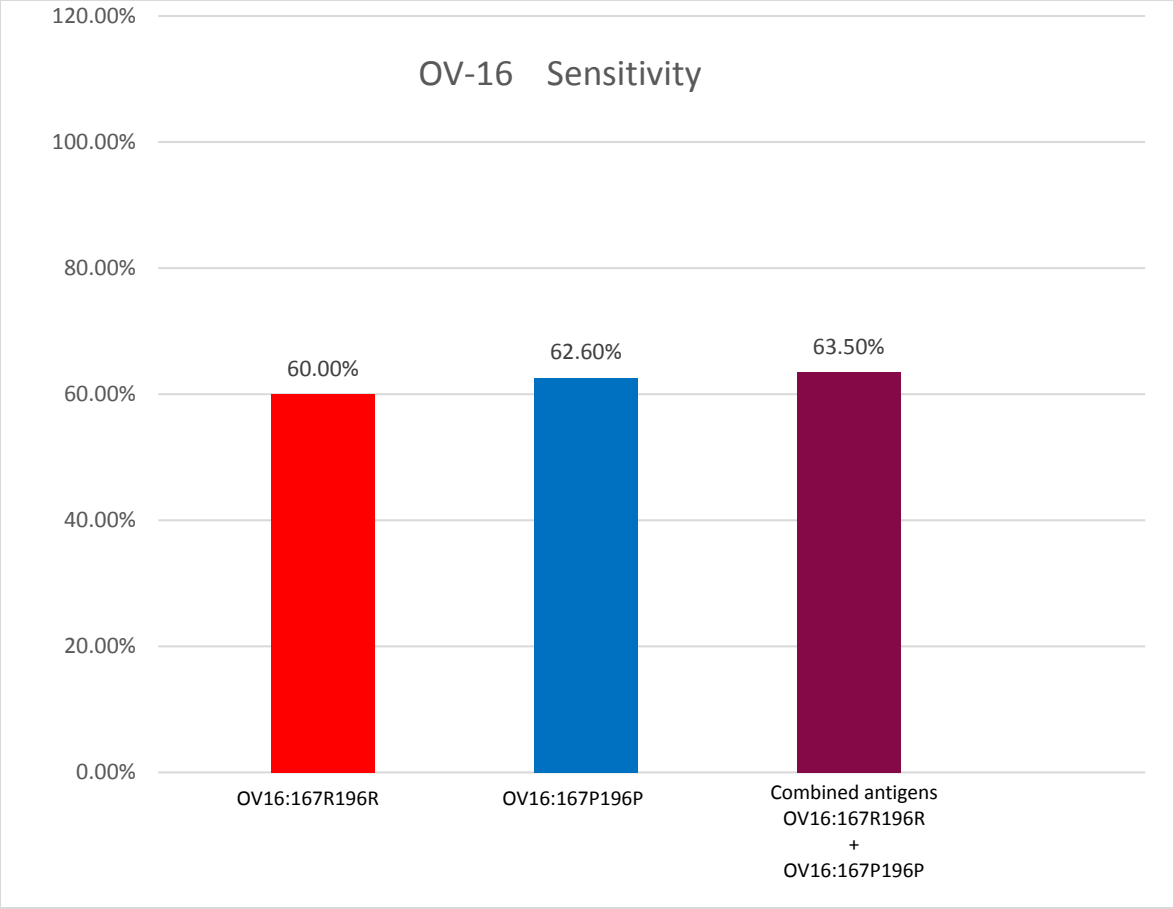


Figure 7: OV-16 ELISA; sensitivity comparisons of antigenic polymorphisms

Specificity was not established during this research as all 704 serum samples that were tested via Ov-16 ELISA were from individuals with known positive skin snips indicative of infection with *O. volvulus.*

OV-16 ELISA GST Cross-Reactivity

All the serum samples tested negative for the presence of GST via Ov-16 ELISA upon evaluation of signal at 405nm. It was therefore determined that no cross reactivity with GST exists in any of the 704 serum samples that were tested indicating that all Ov-16 positive values represent true positives for the Ov-16 portion of the fusion protein. Only *O. volvulus* was analyzed in this research, therefore it is unknown if there may be cross-reactivity with other filarial parasites and/or other *Onchocerca spp*. that could be present in the region where these sera were collected.

CHAPTER FOUR:

DISCUSSION

Control programs rely on OV–16 ELISA as a diagnostic tool to evaluate the progress towards elimination of onchocerciasis, which is currently ongoing primarily through mass distribution of ivermectin, supplemented in some places with black fly habitat targeted control efforts (Cupp, et al., 2012). To maintain quality control and improve upon the Ov-16 ELISA format it is essential to evaluate the tests used for verifying elimination, including use of the recently developed humanized monoclonal antibody positive control. The importance of developing monoclonal antibodies for positive controls in OV-16 ELISA cannot be stressed enough. As onchocerciasis elimination efforts continue, it becomes increasingly important to have a set of standard reagents that can be utilized as positive controls. Availability of monoclonal antibody standards have an advantage in that; with the limited availability of pooled positive sera monoclonal antibodies have the potential to be effectively mass produced such that any need could be met. Monoclonal antibody applications could also universalize assay performance across the globe, making results in different regions relatable for data comparisons. Furthermore, these synthetic humanized antibodies could help protect individuals performing these tests from the risk of other infectious diseases which may also be present in the population where pooled sera might be selected, as it avoids the need of finding positive controls within a given foci which also may become harder to

acquire as elimination efforts continue. This helps validate why a humanized monoclonal antibody is a desired choice over pooled sera for positive controls. Furthermore, the more we learn about genetics we may find that research is no longer able to use a sample set of pooled sera from one region of the world to test another region with the same accuracy.

In this research, antigenic polymorphisms did express concern and possible limitations that exist when using a humanized monoclonal antibody for positive controls. Ov-16 antigens Ov-16:167P196P and Ov-16:167P196R were not appropriately recognized by the humanized monoclonal antibody due to unsuccessful epitope binding. The parental construct Ov-16 antigen (Ov-16:167R196R) and the one containing the single mutation at 196R>P (Ov-16:167R196P) demonstrated equivalent binding affinity with the monoclonal antibody. This suggests that the polymorphism occurring at 167R>P disrupted the epitope that the monoclonal antibody recognized. There may be further concern as to whether or not the monoclonal antibody is able to identify the immunodominant epitope of interest given the possibility of other Ov-16 antigenic polymorphisms that may exist in the designing of Ov-16 coating antigens in the ELISA platform. Another proposal that may be of value would be to combine the parental construct Ov-16 antigen (Ov-16:167R196R) with the one which contained both mutations (Ov-16:167P196P), whereby creating multiple binding sites when using the humanized monoclonal antibody standards.

When evaluating the polymorphisms that were detected via the Unnasch laboratory, there was only a slight discrepancy between the sensitivity of the four experimental Ov-16 antigens when evaluating the 704 sera from Liberia and Ghana.

However, it would be interesting to see if these mutated Ov-16 antigens would have a different effect when tested in other foci in varied regions where onchocerciasis is endemic. Another quandary to consider is the possibility that these types of polymorphisms may be occurring with regards to other *O. volvulus* parasites in different regions of the world which may also require evaluation of differing antigenic polymorphisms than those detected through this research. Furthermore, it may be necessary to delve into future effects of antigenic polymorphisms with regards to OV-16 when designing synthetic monoclonal antibody controls, as well as when testing sera in varying foci in *O. volvulus* endemic regions, as variations could potentially be occurring in different foci similar to those noted in this research which may cause difficulty in creating universalized standards for Ov-16 ELISA formats.

To provide consistent evaluation of the ODs for each of the Ov-16 antigens, cut-off values were standardized on a plate to plate basis at a range of 0.01 – 0.12 based on OD readings of the monoclonal antibody that was plated with Ov-16:167R196R (parental construct) and a set of sera tested against the four experimental antigens on a single plate. In this research, all the sera tested were from *O. volvulus* infected individuals and therefore the cut-offs were set conservatively, standard Ov-16 ELISA protocol typically sets cut-off values based on standards at 1:1280 with a mean OD at approximately 0.13 and a range between 0.06 – 0.19, evaluation at a standard cut-off of 0.2 did not appear to change the sensitivity of the results in this investigation. Reactivity of the four experimental antigens with regards to patient sera also displayed a few outliers that may have indicated other selective epitope binding was at work. A few of these outliers had elevated optical densities well beyond the Ov-16:167R196R (parental

construct) and would be interesting to investigate further, as the antibody-antigen binding affinity occurring in these *O. volvulus* positive samples may assist with future test design applications to potentially increase sensitivity. When evaluating the overall sensitivity of the Ov-16 ELISA against the 704 serum samples from *O. volvulus* infected patients, the results were consistent with current Ov-16 ELISA applications that are in place in the field. Although the combined effort of Ov-16:167R196R (parental construct) and Ov-16:167P196P (containing both mutations) did not indicate statistical significance exists for increased sensitivity, the percentage difference could prove to be statistically sound given further evaluation with a larger sample size than that which was applied in this investigation (n= 704). The sensitivities of 60.0% in Ov-16:167R196R (parental construct), 62.6% in Ov-16:167P196P (containing both mutations), and at best 63.5% when combined, illustrated that there is always work to be done in order to provide the best possible screening capabilities with regards to Ov-16 ELISA and onchocerciasis elimination. As the sera obtained for use in this investigation was acquired from *O. volvulus* infected individuals in foci from Liberia and Ghana only, another avenue to extend future studies regarding these particular Ov-16 antigenic polymorphisms would be through multi-facility collaborative research efforts on *O. volvulus* positive sera from other foci in various endemic regions, such as east Africa; Uganda and Sudan, or possibly Yemen, that have been maintained in alternative research laboratory collections.

The distribution of onchocerciasis has reduced significantly since the 1980's due to the ongoing efforts of MDA programs (Cupp, et al., 2012). Although only minor differences were noted in the positive test results between the four experimental Ov-16

antigens, research of antigenic polymorphisms regarding Ov-16 ELISA may be essential to address the implications that can occur with current elimination efforts if such mutations allow false negative test results within regions of the world thought to be at or near elimination status. Monoclonal antibody controls should be researched further to account for potential antigenic polymorphisms that are occurring in the various foci affected by onchocerciasis which may limit the ability for these controls to work appropriately in Ov-16 ELISA formats when such polymorphisms are present in the coating antigen.

To date there are still many challenges to face in the journey to onchocerciasis elimination, including the continued exploration for proficient diagnostic tools, even without the event of Ov-16 antigenic polymorphisms. There is also a growing need to explore drug alternatives; to treat high risk cases that have co-endemicity infections like *Loa loa*, to prepare for the event of possible ivermectin resistance, and to take a deeper look into macrofilaricides to rid the endemic population of the adult parasites which have the ability to perpetuate the cycle. Continued habitat targeting control methods, possibly aided through the use of GIS, and pooled black fly testing to detect the presence of the parasite within the fly should also be investigated to ensure effective elimination strategies are underway. Prior onchocerciasis research has paved the road in establishing the techniques and treatments that are currently used to control the disease, the path has certainly revealed continued research is necessary to build upon these accomplishments with more field appropriate test applications that have the desired specificity and sensitivity to detect recent infections in order to determine if

transmission is occurring, if reemergence in previously interrupted foci exists, or if disease interruption or elimination status has been achieved within a given foci.

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