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Victoria Lee Walsh April 18th, 2016

Evaluating the Performance of Two Serologic Tests for Detection of Onchocerciasis in Two Hyperendemic Regions

By

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Degree to be awarded: Master of Public Health

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Evaluating the Performance of Two Serologic Tests for Detection of Onchocerciasis in Two Hyperendemic Regions

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B.S.
Berry College
2014

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Abstract

Evaluating the Performance of Two Serologic Tests for Detection of Onchocerciasis in Two Hyperendemic Regions

By Victoria Lee Walsh

Background: Onchocerciasis is a leading cause of blindness globally, with over 37 million individuals currently infected, and has been targeted for elimination.

Methods: A secondary analysis was performed on data from Ethiopia and Uganda to increase understanding of the diagnostic accuracy of Ov16 IgG4 enzyme-linked immunosorbent assay (ELISA) and Ov33 IgG4 multiplex bead assay (MBA) in a hyper-endemic setting. A standardized questionnaire was used to collect demographic data, a physical exam was performed to assess onchocerciasis-related eye and skin disease and other filarial infections, and skin biopsies and blood samples were collected for laboratory diagnostics. Chi-square and median tests were performed to assess for differences in covariates across country of origin. Logistic regression was performed to identify covariates significantly associated with a positive Ov16 IgG4 ELISA, controlling for country. Stepwise multivariable model selection identified covariates to include in latent class analysis (LCA). The latent class model included *a priori* and significant covariates, and the following diagnostic tests: skin snip microscopy, real-time polymerase chain reaction (qPCR), Ov16 IgG4 ELISA, Ov33 MBA, and Ov17 MBA to inform latent class assignment.

Results: There were 1,000 enrollees—nine were excluded for incomplete diagnostic data (N=991). The median age was 39.5 years old, and 47.3% of participants (n=469) were male. There were 774 (78.1%) Ov16 IgG4 ELISA positive individuals, 800 (80.7%) Ov16 MBA positive, 819 (82.6%) Ov33 MBA positive, and 634 (64%) Ov17 MBA positive. There were 147 (14.8%) skin snip microscopy positive individuals, 209 (21.1%) PCR positive, and 225 (22.7%) qPCR positive. Using skin snip microscopy and qPCR as the referent group, estimated sensitivities were 92.7% and 94.0%, and specificities were 26.3% and 20.9% for Ov16 IgG4 ELISA and Ov33 MBA respectively. The following covariates were included in the final model: participant's age, sex, occupation as a farmer, and presence of skin nodules. LCA estimates of specificity were 79.4% and 70.4% for Ov16 IgG4 ELISA and Ov33 MBA respectively.

Conclusions: Serologic tests are better at identifying patent infections than parasitologic methods would suggest given that estimated specificities for Ov16 IgG4 ELISA and Ov33 MBA were three-fold higher using LCA compared with skin snip results.

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Chapter I. Literature Review

I. Onchocerciasis Introduction

A. Global Burden of Onchocerciasis

Onchocerciasis, or river blindness, is one of the leading infectious causes of blindness globally. Approximately 37 million individuals are currently affected by river blindness and an estimated 123 million people living in Sub-Saharan Africa, the Americas and Yemen are at risk for infection (reviewed in [1]). The 31 endemic countries in Africa represent the greatest burden of disease, where 99% of currently infected individuals reside (reviewed in [2]). In addition, there are 6 foci in the Americas, and several foci in Yemen where onchocerciasis is endemic (discussed in [3]).

B. Parasite Biology and Lifecycle

Onchocerca volvulus (*O. volvulus*) is a filarial nematode responsible for onchocerciasis. The lifecycle of *O. volvulus* involves a blackfly vector and a human host. Blackflies of the genus *Simulium* have a preference for breeding along fast-flowing water, thus the greatest risk of infection occurs in individuals living near moving bodies of water (reviewed in [4, 5]). Multiple bites are often required for successful transmission of the infectious third larval stage (L3) to the human host [6, 7]. Once inside of the host, the L3 mature into adult worms that typically reside in the subcutaneous connective tissue. Thousands of larvae, called microfilariae (mf), are produced each day by mature females for a range of nine to twelve years equating to millions of mf produced in a host (discussed in [6-9]). These mf typically live 1-2 years in the human body during which time they can be ingested by another blackfly [6]. The lifecycle is completed when the mf migrate through the fly and mature into L3 ready to infect their next human host [6].

C. Disease Pathology/Morbidity

Individuals infected with *O. volvulus* present with a range of skin symptoms including severe itching, dermatitis, and depigmentation and eye symptoms including decreased visual acuity and blindness (discussed in [10-12]). The variation in pathology observed in infected individuals can be attributed to a variety of factors including host genetics and the *O. volvulus* strain that the individual is infected with (discussed in [13]). The two genetically distinct strains of *O. volvulus* vary in the severity of pathology observed; infection with the savannah strain results in much higher risk for blindness than infection with the forest strain (reviewed in [14-17]). The difference in concentration of *Wolbachia,* an endosymbiotic bacteria, between the two strains may serve as an additional risk factor in the severity of ocular disease given that *Wolbachia* proteins trigger inflammatory responses in the hosts [18, 19]. Skin nodules, known as onchocercomata, may also form around adult worms as a result of the host's immune system attempting to wall off the adult worms [6]. They are typically found on the limbs, above the iliac crest, and on other bony prominences, and serve as a visual or palpable indicator of infection (discussed in [20, 21]).

II. Treatment of Onchocerciasis

A. Current Treatment

Current MDA of river blindness is based on the use of ivermectin (IVM). IVM was first developed in the mid-1970s as a member of the family of anthelminthic drugs known as avermectins and approved for treatment in humans in the 1980s [22]. These products of microbial fermentation were derived from an actinomycete as described by Burg and colleagues, and can target a range of parasitic infections [22]. IVM is the only avermectin

that has been demonstrated to be efficacious in targeting a variety of parasitic infections ranging from ectoparasites to internal helminthic infections, such as onchocerciasis [23]. IVM acts as a microfilaricide, which means that it kills the mf circulating in the infected patient (discussed in [24]). IVM is also safer than diethylcarbamzine (DEC) and suramin, two previously identified treatments that have adverse side effects (discussed in [25]). This was supported by a double-blind randomized control trial conducted in Senegal by Diallo and colleagues in which IVM was found to have minimal adverse side effects compared to DEC and a more prolonged decrease in mf density [26]. One additional study and a meta-analysis of the available evidence demonstrated that once annual treatment with IVM reduced the symptoms of onchocercal skin disease and prevented blindness [27, 28].

B. Future Directions in Treatment

Research is ongoing for the development of drugs that kill adult worms—macrofilarcides—due to concerns about *O. volvulus* developing resistance to IVM (addressed in [29]). The development of a macrofilaricidal drug could also accelerate programs progress towards elimination, given that it interrupts the transmission cycle by targeting the source of mf. Macrofilaricidal properties have been demonstrated in doxycycline making it a valid treatment for onchocerciasis (discussed in [25, 30-32]). Treatment with doxycycline has been shown to sterilize female worms by killing the endosymbiotic bacteria living in the adult worms [25, 30-34]. Doxycycline can also kill adult worms with an observed efficacy of up to 80% [35]. Compared with IVM alone, macrofilaricidal drugs have the potential to more rapidly decrease the prevalence of infection and interrupt the cycle of transmission [25].

III. Elimination of Onchocerciasis

A. Theory and Conceptual Framework for Elimination

Initial efforts to address onchocerciasis as a public health problem focused on control of the morbidity caused by river blindness in areas at highest risk for blindness. This was accomplished via long-term vector control. As programs progressed and IVM became available, it became apparent elimination of the disease might be possible. It has been demonstrated that treatment with IVM reduces mf loads in the community and over time the number of infected black flies decreases, thus providing a scenario for interruption of transmission [36]. In principle the interruption of transmission will prevent any new infections from occurring and with sufficient time active infections will resolve as adult worms die off, allowing for elimination of onchocerciasis. Furthermore, the open-ended commitment made by Merck in 1987 to donate IVM enabled efforts to treat all affected communities to be dramatically scaled up making elimination a more attainable goal for programs [24, 37].

The World Health Organization (WHO) has identified the following three stages of programs as they progress towards elimination of onchocerciasis: 1) suppression of transmission, which is characterized by a lack of introduction of new infective larvae into the human population but risk of an increase parasite transmission of treatment is discontinued; 2) interruption of transmission, which occurs when the parasite population cannot increase in the human population even if treatment is discontinued; 3) and lastly elimination of transmission, which is verified via a three-to-five-year surveillance period in which no active transmission has occurred in the absence of ongoing treatment [37, 38]. WHO established the following set of criteria for elimination of morbidity and

interruption of transmission of human onchocerciasis: demonstration of the absence of reversible lesions in the anterior chamber of the eye; demonstrations of the absence or near absence of infective-stage larvae of *O. volvulus* in the vector population using *O. volvulus*-specific PCR, with a minimum of 10,000 flies sampled; demonstration of the absence of detectable infection (as evidenced by mf, nodules, immunologic, or other proven tests) in untreated children reaching the age of five; demonstration of the absence of detectable infection (as evidenced by mf, nodules, immunologic, or other proven tests) in untreated, new residents who have migrated into an endemic area where transmission has been interrupted [36, 38]. For each of the criteria for absence of detectable infections, a five-year cumulative incidence rate of less than one new case per 1,000 is considered acceptable [36, 38]. These criteria were operationalized by programs in the Americas, and new criteria have recently been released.

B. Empiric Evidence for Elimination

Initial evidence for the possibility of elimination of onchocerciasis came from the Americas. Efforts for onchocerciasis control had been ongoing since the mid-20th century and were scaled up in 2000 with the introduction of semiannual IVM MDA [36]. Following several years of semiannual IVM treatment in Guatemala, evaluation of the interruption of transmission was conducted in 2006 [36]. Adapting the WHO elimination criteria described earlier, the program demonstrated the absence of *O. volvulus* in the vectors using PCR and demonstrated the absence of infection in children by conducting Ov16 IgG4 ELISAs in school children (ages six to twelve years old) [36]. Ov16 serology was used because a negative assay is indicative of a lack of infection with *O. volvulus* during the child's lifetime, so it was felt that its use would be indicative of a lack of

transmission.[36]. In addition to the successes observed in Guatemala, elimination has been demonstrated in Mexico, Colombia, and Ecuador [3, 11, 39-42]. As the progress report for elimination in the Americas highlighted, interruption of transmission has been achieved in eleven out of the thirteen foci and several of the once endemic regions have reached elimination through the efforts of the Onchocerciasis Elimination Program in the Americas (OEPA) and collaborating partners [37].

The elimination of river blindness in Africa, as compared to the situation in the Americas, is complicated by the presence of multiple filarial infections in the same area that make detection and treatment difficult, a weaker infrastructure in place to ensure consistently high coverage with IVM MDA, and the existence of many foci of infection in close proximity to one another. In spite of these concerns, several foci in Africa have demonstrated that elimination is feasible with IVM MDA. Diawara and colleagues provided evidence for successful elimination of onchocerciasis in Senegal and Mali following fifteen to seventeen years of MDA [43]. The potential for elimination in Africa has been demonstrated from studies conducted in Uganda [44-46], Sudan [47], and Nigeria [48, 49]. Collectively, the successes of these countries have been critical in encouraging the African control programs to move forward with the goal of eliminating onchocerciasis.

C. Modeling to Determine Feasibility

As evidenced by the success the Americas, what once seemed unattainable now is being identified as a goal for many other endemic countries. The exact interventions for elimination of onchocerciasis are unclear as programs adapt their control strategies to elimination and evaluate the likelihood of achieving this new target. One such example is

the programmatic shift by APOC from morbidity control to elimination. Using the ONCHOSIM computer model, Coffeng and colleagues simulated various scenarios manipulating coverage levels and IVM distribution in Africa [50]. Their model suggested that biannual treatment reduced the number of years IVM was needed, which is important for weak programs that may not be able to ensure annual MDA for at least fifteen years [50]. Their results supported prior data that the pre-control mf prevalence and biting density are associated with the number of rounds of MDA needed, which may suggest that vector control is important to elimination programs [36, 51]. Duerr and colleagues used a computer model to identify critical transmission thresholds for elimination following the successes of observed in Senegal and Mali, which predicted that a targeted decrease in the biting rate in conjunction with MDA may accelerate progress towards elimination. Additional modeling suggested that elimination of onchocerciasis based on MDA was feasible with a predicted biting threshold of approximately 730 bites per person per year [52]. Programs with more annual biting rates greater than 730 bites per person may need to implement additional vector control strategies to reach elimination goals.

Alley and colleagues sought to model the feasibility of achieving successful elimination given the development of a macrofilaricidal drug that could be used in mass drug administration [25]. In providing justification for their model, the authors discussed the concerns about long-term IVM MDA—geographic coverage will never reach 100%, low level of transmission may be ongoing, potential development of IVM-resistance, and the political will and coordination required to ensure MDA continues for the length of the adult worm lifespan. Based on the conclusions of their model, macrofilaricidal drugs are

much stronger candidates for achieving complete elimination of onchocerciasis [25]. However, as noted by Coffeng and colleagues, regardless of whether a macrofilarcide or a microfilaricide is being used high coverage levels are necessary in order to achieve successful elimination of onchocerciasis given the length of the adult lifespan and the potential for recrudescence if low levels of transmission are not interrupted [25, 51].

D. Programs for Control and Elimination of Onchocerciasis

There are three key programs that share in the history of onchocerciasis control and elimination: the Onchocerciasis Control Program (OCP), the African Program for Onchocerciasis Control (APOC), and the Onchocerciasis Elimination Program for the Americas (OEPA). OEPA was established in an effort to eliminate onchocerciasis in the following countries—Brazil, Colombia, Ecuador, Guatemala, Mexico and Venezuela [53]. In contrast, OCP and APOC were charged with onchocerciasis control in the endemic regions of sub-Saharan Africa. The progress and history of each of the programs is profound, as they have made incredible strides in their goals to reduce the suffering and morbidity associated with this disease, and it is important to learn from their successes moving forward to reach the goal of global elimination of onchocerciasis.

OEPA was first established in 1991 in response to the Pan American Health Organization's (PAHO) resolution to eliminate onchocerciasis as a public health problem from the Americas by 2007 [37, 53]. The strategy employed by OEPA was two-pronged: distribute IVM every six months in areas of documented transmission and provide health education and encourage community involvement. OEPA was responsible for establishing baseline data for the burden of disease at the beginning of the program that was used for analyses of program impact [53]. The characterization of the burden in this

region highlighted epidemiological differences compared with the distribution of onchocerciasis in Africa. The success of this program has been impressive with active transmission currently occurring in only two foci along the border of Venezuela and Brazil [37, 53].

OCP began operations in 1975, initially targeting seven countries in Africa with the objective of controlling onchocerciasis to reduce the incidence of blindness [54]. By the close of the program in 2002, eleven countries had been included in their operational strategies which employed vector control as the primary intervention. The OCP strategy emphasized aerial larvaciding of moving bodies of water in areas with known transmission. This was a heavily resource and time-intensive process involving weekly larvaciding of the affected areas during each transmission season for a minimum of 14 years—the average life span of adult worms. While this strategy offered promising results in the interruption of transmission, the benefits resulting from the decrease in the worm burden were not realized for many years because there was no direct impact on the worms that already infected individuals. It would take many years before the mf density in individuals decreased as the adult worms slowly senesced and died. During the time period of the OCP, the application of IVM as a treatment for onchocerciasis was identified, and it rapidly became integrated into a new approach that led to the development of APOC in 1995. APOC was challenged with the goal of creating sustainable programs for treatment with IVM, specifically targeting communities that had at least 20% prevalence of nodules as established through Rapid Epidemiological Mapping of Onchocerciasis (REMO) [4, 5, 29, 55]. The program was responsible for ensuring that vector control was continued in regions with uninterrupted transmission [4,

5]. Together OCP and APOC have achieved significant milestones in reducing the burden of onchocerciasis in their target countries. Over 100 million have received treatment for onchocerciasis and select foci in several countries have potentially met the elimination threshold [1].

IV. Current Methods of Diagnosis

As with many helminthic infections, the diagnostic measures available to detect onchocerciasis vary in terms of sensitivity, specificity, and field applicability. Historically, onchocerciasis was mapped based on observation of skin nodules and validated using superficial skin biopsies called skin snips [51, 56]. As technology has developed, new diagnostic methods have been put forth in attempts to improve the accuracy of onchocerciasis detection. Each of these methods has varying degrees of improved diagnostic accuracy and time intensity for testing in the field. There is an increased need for highly sensitive and specific diagnostic tests as onchocerciasis control programs have successfully decreased the prevalence of onchocerciasis in many regions. Diagnostic methods used historically to establish estimates of infection for control programs are not robust enough to detect low levels of infection in monitoring and evaluation for the purposes of elimination (reviewed in [20, 21, 29, 57-60]).

A. Parasitologic methods

Parasitologic methods are the gold standard for the identification of infection in an individual and involve detecting the parasite in a skin specimen from the affected individual. The skin snip is a superficial skin biopsy that involves two skin punches from the area above the iliac crest (discussed in [20, 21]). The skin specimens are then incubated in saline at room temperature until they are placed under a microscope to

identify the number of mf that emerged [20, 21]. This count is used to determine the mf density in the patient. The identification of mf in skin snip biopsies has been one of the primary tools used in maintaining surveillance in endemic areas. The sensitivity of skin snip microscopy can be improved by conducting polymerase chain reaction (PCR) or real-time polymerase chain reaction (qPCR) to detect the presence of parasite DNA in the skin (discussed in [20, 21]). Lastly, identification of adult worms from an excised skin nodule can provide evidence of infection with *O. volvulus,* however this technique is rarely used in practice.

B. Clinical Methods

One of the primary clinical features that have been used globally as a rapid indicator of onchocerciasis infection is palpation of skin nodules—or onchocercomata. The OCP and subsequently APOC used REMO—a three step process of assessment using nodule palpation, which was less invasive and time intensive compared to skin biopsies, to estimate the burden of onchocerciasis in medium to high prevalence regions [4, 5, 51]. Since the initial mapping using REMO, monitoring and surveillance programs have relied on determining mf density in skin snips, which is not necessarily comparable to nodule palpation. Coffeng and colleagues developed a model that relates nodule prevalence as identified through REMO to estimates of pre-control mf density in the community [51].

While not exclusively a means of clinical diagnosis, indirect detection of mf can be determined through clinical observation of a dermal response to a DEC patch, where irritation and redness would indicate the presence of mf [20]. However, this method is less sensitive and cross-reacts with a similar filarial parasite, *Loa loa,* (discussed in [20, 21, 60]). Additionally, the resulting inflammation and discomfort is not appealing to

individuals. Lastly, observation of skin pathologies known to be associated with onchocerciasis—such as leopard skin or similar depigmentation—in areas with known or anticipated infections, may occasionally be used as an indicator of long term infection (discussed in [61]).

C. Serologic Methods

Introduction to serologic methods

Methods of onchocerciasis detection based on serology provide highly sensitive results that may reduce the concern of insufficient detection of cases in low prevalence regions by using clinical and parasitologic methods. Serologic methods for onchocerciasis detection are based on the detection of antibodies, such as Ov16 and Ov33, present in the sera of an infected individual. These methods typically involve collection of a blood, either a whole blood sample or a serum sample. Continued improvements to serologic measures can aid in the rapid detection and estimation of onchocerciasis burden in areas with annual or biannual IVM mass drug administration (MDA) as the global programs continue to move closer to their target of global elimination (to be addressed in the *Elimination of Onchocerciasis* section).

Summary of Published Studies

Key Antigen-Based Serologic Assays. Multiple antigens have been explored to determine whether they could be used as the basis of a diagnostic test for river blindness. One of the most pivotal studies relevant to the current method of serologic diagnosis was the isolation and characterization of the *O. volvulus* Ov16 antigen by Lobos and colleagues [62]. Ov16 is an antigen specific to adult worms, and is found in the hypodermis, cuticle and uterus of female adult worms. The highly specific nature of

Ov16 to *O. volvulus* makes it particularly useful as a diagnostic test. The initial evaluation of IgG4 antibody to this antigen was in an enzyme-linked immunosorbent assay (ELISA) that was used to evaluate sera from patients with known onchocerciasis infections from endemic regions in Mali and reported sensitivity of 90% and specificity of 98% [62].

In addition to Ov16, Ov33 and Ov17 have been investigated for onchocerciasis detection. Characterization of the Ov17 antigen was conducted by Bradley and colleagues [63]. It was demonstrated that Ov17 has the potential to be a marker of active infection with *O. volvulus* given that it is found in three stages of the lifecycle—the adult stage, the mature mf stage and the L3 larval stage [63]. When evaluated in sera from onchocerciasis positive individuals in Uganda and Ethiopia, Ov17 had sensitivities of 86.2% and 76.1% for IgG and IgG4 assays respectively (2016, Feeser et al., in press). Sera positive for human parasites other than *O. volvulus* were obtained from the following countries non-endemic for onchocerciasis: Haiti, Kenya, Brazil, India, Bangladesh, Sri Lanka, Tahiti, Indonesia, United States, Peru, Argentina, and Mali, in order to evaluate the specificity of Ov17 (2016, Feeser et al., in press). The reported specificities were 79.2% and 82.8% for IgG and IgG4 assays respectively, with cross-reactivity with lymphatic filariasis noted (2016, Feeser et al., in press). Similarly to Ov17, Ov33 is a recombinant antigen whose natural homologue is found in nearly all stages of the parasite lifecycle. Lucius and colleagues demonstrated that Ov33 had a sensitivity of 96% in a population of confirmed onchocerciasis patients from Cameroon, Mali, and Guatemala [64]. The specificity of Ov33 was reported at 100% when evaluated with specimens from individuals infected with *Brugia malayi* and *Dirofilaria immitis*, suggesting that it was a promising candidate

for application in diagnostic assays [64]. Furthermore, the specificity of the IgG4 assay using Ov33 when tested in the same negative control population as the Ov17 IgG and IgG4 tests was 98.6% (2016, Feeser et al., in press).

Other Antigen-based serologic assays. Andrews and colleagues investigated the role of recombinant antigens for use in ELISA in non-endemic regions for onchocerciasis detection, based on a similar study design conducted by Bradley and colleagues [65, 66]. The recombinant antigens they evaluated were derived from combinations of Ov20, a glycoprotein secreted in the intestinal wall of female adult worms [65]. Overall sensitivity observed for ELISA assay when tested in two endemic populations in Cameroon and Guatemala with a confirmed clinical diagnosis of onchocerciasis were 93.2% and 93.5% respectively [65]. Chandrashekar and colleagues identified the following two recombinant antigens—OC 3.6 and OC 9.3—as fairly sensitive for use in ELISA detection of infection (OC 3.6-93%, OC 9.3-84%, combined-98%) when tested using sera from endemic regions in Guatemala, West Africa, and Ecuador [67].

Ov16-based Rapid Diagnostic Tests. Lipner and colleagues characterized an immunochromatographic card test (ICT) developed to allow for rapid detection in the field of anti-Ov16 antibodies in endemic populations in Burkina Faso and Coˆte d'Ivoire [57]. The sensitivities observed were fairly high, 81.1% and 76.5%, and specificity was 100%, providing evidence for the use of the ICT card in the field [57]. The company that developed this test never produced it for large-scale use and eventually the product was cancelled. Fortunately, a new rapid diagnostic test (RDT) was developed by PATH using the Ov16 antigen with reported sensitivity of 89.1% and specificity of 97%, which was lower than originally reported [68]. The test was evaluated in 449 specimens from

endemic and non-endemic regions. Mf-positive samples were from Ghana, Liberia, and United States (US) travelers [68]. Onchocerciasis negative sera included specimens with no parasitologic evidence of parasites in Liberia, Mali, Guatemala, Ecuador, and the US blood bank, as well as specimens positive for other filarial infections from the Cook Islands, India and US travelers [68].

D. Strengths and Limitations of Diagnostic Methods

Evaluating the strengths and limitations of diagnostic methods helps to ensure the appropriate methods are used to monitor progress towards elimination of onchocerciasis. While nodule palpation is a rapid means of assessment, it is has low specificity which can result in misdiagnosis of other conditions including lipomas or cysticercosis, particularly in low prevalence settings [51]. Comparatively, skin snip microscopy and PCR are more specific and function well for pre-treatment evaluations. However, skin biopsies are invasive and may be painful for patients, and may also place individuals at risk for a secondary infection or transmission of blood-borne pathogens. Due to these concerns, community preference for these diagnostic methods is limited (discussed in [20, 21, 60]). Skin snips may result in false negatives for light infections or, given that MDA suppresses mf for up to one year, in areas with ongoing MDA [20, 21]. Serologic tests are not influenced be MDA and can be conducted at any time during program monitoring unlike skin snips that must occur one year following the last MDA. They can provide rapid results when card tests are used, but are more time and resource intensive when ELISA is used. Serologic tests cannot differentiate between past and active infections, unless seropositivity disappears rapidly after cure [20]. However, if used in a targeted manner such as restricting to particular age groups serologic tests can identify active

infections (discussed in [20, 21]). Serologic tests would be most appropriate in when estimating burden in regions where MDA has not been occurring consistently or has had low coverage. They can also be used to determine if transmission has been interrupted by testing individuals who moved into the area or were born after it was believed transmission had been interrupted. Negative results in these populations indicate that there has been no recent infection.

V. Latent Class Analysis

Evaluation of the various diagnostic tests for onchocerciasis in the context of elimination using IVM highlights the lack of gold standard method for detection of infection. Statistical methods such as latent class analysis (LCA) have been used to evaluate the sensitivity and specificity of diagnostic tests when traditional gold standards are not available [69-72]. In the context of assessing diagnostic accuracy, LCA typically assumes that each of the tests are independent of one another, in other words, that observed results in one test do not determine or influence the results observed from another test [69, 70, 72]. The key principle of LCA is that there is an underlying class, as defined by the combination of tests that represents true disease status that cannot be measured with imperfect diagnostic tests. By combining the available non-gold-standard tests simulated "latent"—or true disease status—classes can be modeled. These latent classes can be used to estimate sensitivities and specificities for individual diagnostic tests using the predicted probability of a positive outcome (infection with *O. volvulus* in this analysis) [69, 70].

Boelaert and colleagues evaluated the role of LCA in validating available diagnostic tests for *Leishmania infantum* infections in canines [72]. The authors included

five variables of interest—clinical status, parasitologic identification of *L. infantum* on a smear, indirect immunofluorescence antibody test (IFAT), ELISA, and direct agglutination assay (DAT)—and used a two latent class model which dichotomized the outcome variable as infected or non-infected. The authors evaluated a three-class model, which considered healthy uninfected, asymptomatic, and ill as the three outcomes, but determined that the two-latent-class model fit the data better. Estimates of sensitivity and specificity using LCA were compared with respective estimates using 2x2 contingency tables with parasitologic results as truth. The model indicated that there was a higher prevalence of infection and parasitologic identification was less sensitive than previously believed, and that the estimates for other diagnostic tests were more precise using LCA than with the 2x2 contingency tables [72]. In a similar study, Machado de Assis and colleagues evaluated IFAT, DAT, ELISA, microscopy and a rapid test for visceral leishmaniasis in Brazil using LCA. The latent class model provided estimates of sensitivity and specificity for each diagnostic test, and their results indicated that parasitologic evidence was not a sufficient reference standard to estimate sensitivity and specificity of other diagnostic tests [73].

Tustin and colleagues, who theorized that adding covariates to LCA would improve the model as it would incorporate a combination of unique clinical factors in addition to test results, compared LCA models with and without covariates in an evaluation of tests for *Trypanosoma cruzi* [70]. They found that including covariates improved the predictive value of the LCA model [70]. The basic methodologies for LCA described above can be applied to evaluation of diagnostic tests for onchocerciasis where

the goal remains to estimate the model parameters of sensitivity and specificity where the true prevalence of infection is unknown.

Need, Goal, Aims:

As global programs continue working towards the goal of eliminating morbidity and transmission of human onchocerciasis, there is a need to better understand the performance of serologic tests used to identify onchocerciasis. Maximizing the specificity of diagnostic tests in regions with low prevalence or ongoing MDA will allow for a measurable threshold for elimination of human onchocerciasis. The goal of this thesis is to increase understanding of the diagnostic accuracy of the Ov16 IgG4 enzyme-linked immunosorbent assay (ELISA) and the Ov33 multiplex bead assay (MBA) in a hyper-endemic setting. In order to achieve this goal the following aims will be met:

1. Identification of a priori clinical predictors of positive onchocerciasis infection.
2. Identification of non-serologic predictors of positive Ov16 antibody test using logistic regression
3. Assessment of the sensitivity and specificity of the Ov16 and Ov33 antibody tests independently using LCA
4. Tentative: Assessment of the sensitivity and specificity of the Ov16 and Ov33 antibody test combined using LCA to determine if adding Ov33 testing increases the probability of detecting onchocerciasis positive individuals.

Significance:

As programs are moving forward in their elimination goals, an issue regarding the specificity of the test has arisen. The criteria for elimination is less than one new case per 1,000 individuals; however, the current methods of detection are only approximately 98%

specific thus countries will not continually fall short of the elimination threshold on the basis of potential false positives alone [38, 68]. Using LCA to estimate the specificity of Ov16 IgG4 ELISA and Ov33 MBA may reduce the number of false positive test results when evaluating whether transmission has been interrupted. Current guidelines require skin biopsies be performed on all serologically positive individuals immediately and after one and a half years which is time intensive and difficult to ensure that individuals are not lost to follow up. The availability of a second diagnostic serologic test could simplify confirmatory testing of individuals that have positive Ov16 results by allowing for a faster, less invasive alternative to the skin biopsies. Endemic countries in Sub-Saharan Africa that are in the monitoring and evaluation phase of their elimination programs need diagnostic methods that are sufficiently specific to meet the WHO threshold for elimination of transmission of onchocerciasis in humans.

Chapter II. Manuscript

Evaluating the Performance of Two Serologic Tests for Detection of Onchocerciasis in Two Hyperendemic Regions

Abstract

Background: Onchocerciasis is a leading cause of blindness globally, with over 37 million individuals currently infected, and has been targeted for elimination.

Methods: A secondary analysis was performed on data from Ethiopia and Uganda to increase understanding of the diagnostic accuracy of Ov16 IgG4 enzyme-linked immunosorbent assay (ELISA) and Ov33 IgG4 multiplex bead assay (MBA) in a hyper-endemic setting. A standardized questionnaire was used to collect demographic data, a physical exam was performed to assess onchocerciasis-related eye and skin disease and other filarial infections, and skin biopsies and blood samples were collected for laboratory diagnostics. Chi-square and median tests were performed to assess for differences in covariates across country of origin. Logistic regression was performed to identify covariates significantly associated with a positive Ov16 IgG4 ELISA, controlling for country. Stepwise multivariable model selection identified covariates to include in latent class analysis (LCA). The latent class model included *a priori* and significant covariates, and the following diagnostic tests: skin snip microscopy, real-time polymerase chain reaction (qPCR), Ov16 IgG4 ELISA, Ov33 MBA, and Ov17 MBA to inform latent class assignment.

Results: There were 1,000 enrollees—nine were excluded for incomplete diagnostic data (N=991). The median age was 39.5 years old, and 47.3% of participants (n=469) were male. There were 774 (78.1%) Ov16 IgG4 ELISA positive individuals, 800 (80.7%) Ov16 MBA positive, 819 (82.6%) Ov33 MBA positive, and 634 (64%) Ov17 MBA positive. There were 147 (14.8%) skin snip microscopy positive individuals, 209 (21.1%) PCR positive, and 225 (22.7%) qPCR positive. Using skin snip microscopy and qPCR as the referent group, estimated sensitivities were 92.7% and 94.0%, and specificities were 26.3% and 20.9% for Ov16 IgG4 ELISA and Ov33 MBA respectively. The following covariates were included in the final model: participant's age, sex, occupation as a farmer, and presence of skin nodules. LCA estimates of specificity were 79.4% and 70.4% for Ov16 IgG4 ELISA and Ov33 MBA respectively.

Conclusions: Serologic tests are better at identifying patent infections than parasitologic methods would suggest given that estimated specificities for Ov16 IgG4 ELISA and Ov33 MBA were three-fold higher using LCA compared with skin snip results.

Background & Introduction

Onchocerciasis is one of the leading infectious causes of blindness globally with approximately 37 million individuals currently affected. Furthermore, an estimated 123 million people living in Sub-Saharan Africa, the Americas and Yemen are at risk for infection [1]. The filarial nematode *Onchocerca volvulus (O. volvulus*) is the parasite responsible for infection, and is transmitted via blackflies of the genus *Simulium* [4, 5]. Individuals infected with *O. volvulus* present with a range of skin symptoms including severe itching, dermatitis, leopard skin, and depigmentation and eye symptoms including decreased visual acuity and blindness [10, 14].

Initial efforts to address onchocerciasis as a public health problem focused on control of the morbidity caused by river blindness in areas at highest risk for blindness and was accomplished via long-term vector control. As country programs demonstrated successful interruption of transmission and ivermectin (IVM) became widely available, it became apparent elimination of the disease might be possible. It has been demonstrated that treatment with IVM reduces the microfilarial (mf) load in the community and over time the number of infected blackflies decreases, thus providing a scenario for interruption of transmission [36]. Interruption of transmission has led to successful elimination in limited areas in select countries [3, 11, 36, 42].

Elimination programs require robust diagnostic methods for monitoring and evaluation to determine if interruption of transmission has been successful. A variety of diagnostic methods are available to identify onchocerciasis including skin snip microscopy, polymerase chain reaction (PCR), real-time PCR (qPCR), and serologic methods using parasite–derived antigens [20, 21, 62, 74]. Detection of microfilaria (mf)

in the skin through microscopy is the gold standard for diagnosis; however, in regions where IVM mass drug administration (MDA) has been on going the mf prevalence decreases; this method lacks the sensitivity to detect light infections. Serologic tests have been developed in order to detect light infections. An enzyme-linked immunosorbent assay (ELISA) and a rapid diagnostic test (RDT) have been developed using the Ov16 antigen. The Ov16 ELISA has been widely used by elimination programs, while the RDT has only recently been used [62, 68]. However, even with a reported specificity of 98% [62], the expected rate of false positives using Ov16 serology exceeds the number of positives allowed to meet a 95% confidence interval that excludes 0.1% seroprevalence in children less than ten years-old [38].

In order to meet the WHO guidelines for elimination of transmission in humans, endemic countries need diagnostic methodologies that are able to accurately discriminate *O. volvulus* infected individuals from non-infected individuals. The objective of this analysis was to evaluate the diagnostic accuracy of two recombinant antigens—Ov16 and Ov33—in a hyperendemic setting. Latent class analysis (LCA) has been previously used to estimate sensitivity and specificity of diagnostic methods in the absence of a true gold standard [70, 72, 73]. For our purpose, LCA was used to estimate the sensitivity and specificity of Ov16 and Ov33 antigen tests and these results were compared with respective estimates using skin snip results as indicative of true infection status.

Methods

Ethics Statement:

Study protocols were approved by the CDC Institutional Review Board as well as Ugandan and Ethiopian ethical review committees. Adult study participants were enrolled after obtaining informed consent. Children were enrolled after obtaining parental/guardian permission and assent when age-appropriate.

Study Design:

The protocol for the African onchocerciasis specimen bank collection and evaluation of the Ov16 serologic test for *O. volvulus* infection was implemented in 2012 and 2013. The primary objective was to evaluate the performance of the Ov16 IgG4 ELISA in meso-endemic or hyper-endemic regions with limited exposure to IVM by establishing and characterizing a specimen bank with demographic, clinical, parasitologic, and serologic data. A convenience sample of 500 individuals from Ethiopia and Uganda were selected. Participants were eligible if they were at least six years-old and had lived in the village for at least ten years or since birth if younger than ten years-old. Participants from Uganda were enrolled from villages located in the Kitgum and Lamwo districts where annual IVM MDA had begun 3 years prior; however, coverage was low. The last MDA occurred five months prior to study implementation. Participants from Ethiopia were enrolled from villages in the Jimma Zone. Ethiopia rolled out their biannual IVM MDA program five months prior to the study, thus participants may have received one dose of IVM.

Data Collection:

Demographic and risk factor data were collected using a standardized questionnaire. Participants then underwent skin and eye exams followed by skin snip and blood collection.

Demographic variables. In order to obtain detailed information about enrollees, the following data were collected: age, sex, country, village, years of residence in the endemic area, distribution of IVM during MDA in the village, and swallowing the IVM distributed during MDA. Age was reported as a numeric value, and the participant's sex was entered as a dichotomous variable. Villages were documented as text and categorized. Data on participant's occupation was reported; however, the only occupation of interest to this study was farming thus all other occupations were categorized as not farming. IVM distribution and usage for the last year and ever were collected to document the history of IVM for each individual.

Participant reported symptoms. Participants were asked about whether they had experienced itching in the last year, how often they have itching, whether itching disturbs sleep, if they had any skin nodules, and how many were present. Participants were also asked if they had experienced any of the following changes in their skin: thinning of skin, increased wrinkling (not on their face), sagging or drooping of their skin, loss of skin color, leg swelling, inflammation in their leg that seemed to be associated with swelling, and swelling of the testicles in men. They were also asked if they had experienced and of the following changes in their vision: whether they were able to see at all, whether they were able to only see light and dark, whether they were able to see shapes or outlines but not faces or details, whether they experienced blurred vision, whether they were able to

see normally during the day but not at night, and whether they had ever noticed a worm in their eye. With the exception of the number of skin nodules, all variables were treated as binary.

Physical exam. Eye and skin exams were performed by trained professionals. The eye exam included visual acuity and a slit lamp to assess for finding of corneal disease and mf in the anterior chamber of the eye (MFAC). The WHO criteria for functional blindness (visual acuity of 200/20 or weaker) were used to classify individuals as severely visually impaired or not in a binary variable [75]. Data on gross lesions which included whether an eye worm was present and whether the cornea had visible lesions, scarring, or clouding were recorded as binary variables. Punctate keratitis was characterized according to the following: mf that are live/coiled noted in the cornea with no inflammation (punctate keratitis stage A), mf that are straightened/dying in the cornea with no inflammation (punctate keratitis stage B), inflammation around complete mf (punctate keratitis stage C), inflammation around fragmented mf (punctate keratitis stage D), and inflammation only noted with no visible fragments of mf (punctate keratitis stage E). Due to the low prevalence of punctate keratitis in this study population, two binary summary variables were created: any punctate keratitis versus none, and combined stages A and B versus not stages A and B because stages A and B are the most reliable indicators of onchocerciasis-related lesions [76]. The skin exam included an assessment of the presence and number of skin nodules, assessment for signs of onchocercal skin disease (OSD) using the Murdoch et al criteria [12], lymphadenopathy, lymphedema, and hydrocele. Examination for OSD included assessment of the various stages of each of the

following: atrophy (ATR), depigmentation (DPM), acute papular onchodermatitis (APOD), chronic papular dermatitis (CPOD), and lichenified onchodermatitis (LOD).

Specimen Collection and Analysis. Blood specimens were collected from participants using sterile technique in blood collection tubes. Blood was used to make blood smears to assess for the presence of *Mansonella perstans* and *Loa loa,* to run ICT cards to assess for lymphatic filariasis, and to make dried blood spots (DBS) for further analysis at the CDC. The remaining blood was centrifuged to obtain the serum or plasma, which was removed and frozen for further analysis at CDC. Two skin snips were performed on each participant. Snips were taken from the skin over the iliac crest using a Holth corneoscleral punch biopsy tool and incubated in normal saline for twenty-four hours. Snips were then examined microscopically for the presence of mf and placed into preservative and frozen for PCR and qPCR analysis at CDC. Identification of mf in the skin by microscopy in either of the two skin snips was considered a positive skin snip result, and average mf for each participant was calculated by summing the mf from each snip and dividing by two.

Preserved serum and skin specimens were shipped to the CDC and analyzed there. PCR and qPCR assays were conducted to detect the presence of *O. volvulus* genetic material in the preserved skin snips. The methodology for these assays has been previously published by Thiele and colleagues [77]. An ELISA was conducted to assess for the presence of the Ov16 IgG4 antigen using the preserved serum. The protocol for ELISA was a standard ELISA procedure adapted from previously published studies [62]. Preserved serum was diluted, added to the antigen-treated 96-well plates, and screened for an IgG4 antibody response. The serum was also used to detect the presence of Ov16,

Ov33, and/or Ov17 using a multiplex bead assay (MBA). MBA allows for as many as 100 antigens to be simultaneously analyzed in a single specimen using a double signal mechanism that can discriminate and quantify reactivity using a dual detection laser array (2016, Feeser et al., in press). In summary, following a bead-coupling procedure where the onchocerciasis antigens were attached to the SeroMap beads (Luminex Corporation, USA), the preserved serum samples were diluted and added to the 96-well-filtered-bottom plates containing the coupled-beads. After a period of incubation the wells were screened for IgG and IgG4 antibody responses against the target antigens using the dual detection array (2016, Feeser et al., in press). MBA has been used for integrated neglected tropical disease (NTD) surveillance [78-81]. Appropriate cut-points for each of the assays were determined by the lab, and binary variables were created for each of the antigen tests.

Data Management and Data Quality:

Data in the field were collected on PDAs that were programmed with skip patterns and check coding to ensure quality data were obtained. Data were stored in Microsoft Access and imported into SAS 9.3. Lab diagnostic data were entered into Microsoft Excel and imported into SAS 9.3. Data were recoded for analysis purposes, and recoding of variables was checked using frequency tables generated in SAS 9.3.

Data Analysis

All data were analyzed using SAS 9.3 (Cary, NC).

Descriptive statistics:

Frequency tables were used to calculate the count and percentage of the study participants for each dichotomous covariate by country. Sample median and range were estimated for continuous covariates—age, average mf load, and number of skin nodules reported at physical exam—by country as well. Chi-square and Fisher's exact tests were conducted to assess for significant differences in categorical covariates across countries. The primary assumption of chi-square tests is that the expected cell count is at least five, thus for covariates with the majority of the expected cell counts less than five the Fisher's exact test was used. Differences in medians across country of origin were assessed with the median test using PROC NPAR1WAY in SAS 9.3 (Cary, NC). The normality of the distributions of the continuous covariates was assessed using histograms. Ethiopia and Uganda were sampled independently and the size of the populations from each were sufficiently close that the student's t-test is robust to detect differences in variance.

Regression analysis:

Logistic regression was conducted to identify the covariates to be included in latent class analysis (LCA) due to the limitations in the statistical software for LCA which did not allow for selection of covariates. Logistic regression assumes that the observations are independent of one another, which was not met given the design of the cluster sampling used in this study. Too few villages were sampled to meaningfully control for village-level clustering, however it was possible to assess for significant associations between covariates and the Ov16 IgG4 ELISA while controlling for country. A second

assumption of logistic regression is that no extraneous variables are included in the model. Variables without a biologically plausible relationship with a positive Ov16 IgG4 ELISA result were not included in the model. Variables that had a statistically significant association with a positive Ov16 IgG4 ELISA result in bivariable analysis that controlled for country were included in multivariable analysis. Logistic regression also assumes that no important variables are omitted and that the independent variables are measured without error. We felt that these assumptions were reasonably met for the purpose of this model, given that clinical information collected by study personnel were used instead of participant-reported symptoms to improve reliability. Furthermore, the questionnaires and physical examination were designed to collect data most relevant to onchocerciasis to minimize extraneous variables. Independent variables must not be linear combinations of one another in logistic regression; collinearity diagnostics were used in the multivariable model to ensure that this assumption was not violated.

For the purpose of logistic regression, a positive Ov16 IgG4 ELISA was considered to sufficiently indicate true infection, provided that the study areas had not received high coverage levels of IVM MDA (discussed in [20, 21]). Ov16 ELISA results were used instead of Ov16 MBA results given that ELISA is a well-characterized test that has been implemented by country programs, while MBA is a novel technique that is not yet widely available whose cut points for positive and negative are not as easily defined as for ELISA.

Bivariable analysis was conducted to assess for significant associations between the covariates and a positive Ov16 IgG4 ELISA at the alpha = 0.05 significance level, while controlling for country in which the study was performed. This was done because of

statistically significant differences in the prevalence of covariates across countries. Significant covariates in bivariable analysis were included in the initial multivariable model. Age and sex were included in the full model based on *a priori* knowledge of their association with *O. volvulus* infection due to differences in exposure (discussed in [55, 82, 83]). Covariates corresponding to clinical examination findings took priority over participant-reported symptoms—presence of skin nodules, eye findings, and skin findings—when determining which covariates to include in the full model.

Manual stepwise selection with a significance threshold of 0.05 was conducted to identify the final subset of covariates that were strongly associated with a positive Ov16 IgG4 ELISA result. Correlation diagnostics were run on the final model using variance decomposition proportions (VDP) and condition indices (CI) with ≥0.5 and ≥30 cut offs respectively. As a final step to validate the model, bootstrapping was used with a sample size of 200 (with replacement), and the mean estimates and standard errors were calculated to assess model stability (reviewed in [84]).

Latent class analysis (LCA):

LCA using PROC LCA v 1.3.2 (The Methodology Center, Penn State University) was conducted to better assess the sensitivity and specificity of the Ov16 IgG4 ELISA and Ov33 MBA diagnostic tests [85]. The key principle of LCA is that there is an underlying, unobserved factor responsible for the status of outcome—infected or not infected for the purpose of this analysis (reviewed in [69-72]). This factor can be calculated by integrating the categorical variables of interest into a simulated *latent* class which can be used to determine the sensitivity and specificity of diagnostic tests of interest using probabilities. LCA assumes each of the tests included in the model are

independent of one another—that the observed results in one test do not determine or influence the results observed from another test. This assumption was evaluated by assessing changes in model fit when several combinations of diagnostic tests were included in the model. It was identified that including both the PCR and qPCR tests in the model may violate this assumption, thus only qPCR was included in the model. The remaining tests were not shown to violate this assumption and were included in the model. LCA also assumes that every set of responses among the indicators, or tests included in the model, is associated with membership in a latent class. Taking this into account, we limited indicators to those which were strongly associated with infection. Previous characterization of each of the diagnostic tests included in the model has confirmed that they are associated with *O. volvulus* infection; however, consideration of the combination of test results associated with each latent class must be used when drawing conclusions from the model.

The following diagnostic methods were included in the model to define the latent class: skin snip microscopy, qPCR, Ov16 IgG4 ELISA, Ov33 MBA, and Ov17 MBA. Model fit statistics, Akaike's Information Criteria (AIC) and Bayesian Information Criteria (BIC), were used to inform the number of classes to be included by selecting the model with the lowest AIC and BIC. AIC is a statistic that assessed the goodness of fit of the model and the complexity of the model, BIC is a similar statistic that tends to favor more parsimonious models based on the correction for model error used in its computation [86, 87]. Decreases in AIC and BIC from the full model that are ten or more suggest that the more parsimonious model is a better fit. Significant covariates in multivariable analysis were included in the full latent class model to the improve

determination of class assignment. Sensitivity and specificity for Ov16 IgG4 ELISA and Ov33 MBA were estimated using the 2-class latent class model with covariates. Unadjusted sensitivity and specificity for Ov16 IgG4 ELISA and Ov33 MBA were calculated using 2x2 tables with combined skin snip microscopy and qPCR results as the accepted gold standard methods to estimate true infection status. The estimates of sensitivity and specificity calculated using the latent class model were then compared with estimates of sensitivity and specificity using combined skin snip microscopy and qPCR results as truth.

Results

Descriptive statistics:

There were 1,000 individuals enrolled in the study. 991 individuals were included in the analysis, with 9 individuals eliminated due to incomplete diagnostic data (Table 1). The median age was 39.5 years old, and about one half of the study population (n=469, 47.3%) was male. There were 475 (47.9%) individuals that spent most of their day near a river and 315 (319%) lived near a river. The majority (n=800, 80.7%) of the study participants indicated they were farmers. IVM was previously taken by 878 individuals (88.6%), and 652 (65.8%) had taken IVM in the last year. Over two-thirds (n=712, 71.9%) of the participants reported itching in the past year, and 85.4% of those individuals indicated itching disturbed their sleep. Fewer individuals reported skin changes (n=251, 25.3%) and swelling of the leg (n=174, 17.6%). Vision changes were reported in 645 (65.2%) individuals.

In contrast to participant-reported symptoms, there was a much lower prevalence of visual impairment and eye disease attributable to onchocerciasis found during the eye examination. Functional blindness was documented in 73 (7.4%) individuals, and a small percentage of individuals had corneal lesions (n=25, 2.5%) or MFAC (n=29, 2.9%). Any form of punctate keratitis was documented in 32 (3.3%) participants, with punctate keratitis stages A and B in 4 (0.4%) individuals. Approximately one half of the population (n=516, 52.1%) had skin nodules on examination, with a median of one nodule (range 0 to 16 nodules). Depigmentation was the most commonly observed form of OSD documented in 223 (22.7%) participants, and the remaining skin and lymph manifestations of onchocerciasis occurred in less than 20% of the population.

The majority of the study population was seropositive for the antibody response against the antigens of primary interest to this analysis—Ov16 and Ov33. There were 774 (78.1%) participants who had a positive Ov16 IgG4 ELISA, 800 (80.7%) who had a positive Ov16 MBA, 819 (82.6%) who had a positive Ov33 MBA, and 634 (64%) who had a positive Ov17 MBA. Fewer individuals had parasitologic evidence of onchocerciasis: 147 (14.8%) skin snip microscopy positive, 209 (21.1%) PCR positive, and 225 (22.7%) qPCR positive.

The majority of covariates had statistically significant differences across countries using the chi-square, Fisher's exact, and student's t-tests. Fisher's exact test was used for only two variables with expected values less than five: physician documented punctate keratitis stage A and B and physician documented lymphedema in participant. All covariates had statistically significant differences across countries except the following covariates: participant ever took IVM, participant spends most of their day near the river, participant reported changes in their vision, physician documented any punctate keratitis, physician documented punctate keratitis stages A and B, physician documented hanging groin, positive Ov33 MBA, and positive Ov17 MBA.

Regression Analysis:

Of the variables assessed using bivariable logistic regression, the following had a positive significant association with a positive Ov16 IgG4 ELISA result controlling for country: age of participant, male sex, spends most of day or lives near river, farmer, skin nodules at physical exam, number of skin nodules, any OSD with or without skin nodules, skin nodules only (but no OSD), OSD only (but no skin nodules), and skin nodules and OSD combined (Table 2). The variables were included in the full

multivariable model. Following stepwise selection the remaining covariates were determined to have statistically significant associations with Ov16 IgG4 ELISA result controlling for country, and thus appropriate to include in the latent class model: farmer and presence of skin nodules at physical exam (Table 3). *A priori* criteria were used to inform the decision to include age and sex in the final multivariable model as well.

Latent Class Analysis (LCA):

The full latent class model included the following diagnostic tests: skin snip microscopy, qPCR, Ov16 IgG4 ELISA, Ov33 MBA, and Ov17 MBA and controlled for age, sex, presence of skin nodules at exam, farmer, and country of origin. Compared with the two-class model, the three-class model had stronger model fit statistics—AIC values were 439.31 and 53.23, BIC were 493.19 and 136.51 for the two- and three-class models, respectively. However, because the software did not allow for estimation of sensitivity and specificity using a three-class model, the two class model was used for the analysis. Assessment of combinations of diagnostic test results and corresponding individual probabilities for class assignment were used to define latent classes. It was determined that latent class one corresponded to patent infection with *O. volvulus* which included individuals with two or more positive serologic tests and parasitologic positive individuals. Latent class two included individuals with primarily negative serology and negative parasitology and was considered not infected. The estimated specificity for both Ov16 IgG4 ELISA and Ov33 MBA were approximately three-fold higher when the latent class model was used compared with the skin snip results (Table 4). Additionally the LCA estimates for sensitivity were slightly higher as compared with the skin snip result estimates.

Discussion

In the current study, regression analysis and LCA were used to evaluate the performance of serologic tests used to identify onchocerciasis in a hyperendemic setting. Logistic regression identified non-serologic covariates of infection consistent with the known epidemiology of onchocerciasis (discussed in [4, 55]). The multivariable model found that the presence of nodules and participant's occupation as a farmer were significantly associated with a positive Ov16 IgG4 ELISA result. Significant and *a priori* covariates were subsequently used to better define the latent class to improve the probability of class assignment in LCA [70]. Higher sensitivities of Ov16 and Ov33 were demonstrated using LCA compared to parasitologic-based diagnostics. Additionally, the specificities estimated using LCA for Ov16 and Ov33 were nearly three-fold higher than when calculated using parasitologic-based diagnostics. These results indicate that serologic tests are much better at identifying patent infections than parasitologic methods would suggest given that there were significantly fewer false positives than the 2x2 contingency table calculations predicted.

Evaluating sensitivity is a key element of determining the value of diagnostic tests because it quantifies their ability to accurately discriminate between infected and non-infected individuals. Sensitivity is the proportion of truly infected individuals that tested positive out of all of the infected individuals (discussed in [88]). When diagnostic tests are used that have low sensitivity, such as skin snip microscopy, the burden of disease is underestimated and individuals in need of treatment may not be detected (discussed in [20, 21]). Thus for programs preparing to estimate the baseline burden of infection, tests with high sensitivity are preferred to reduce the risk of missing cases. Maximizing the

sensitivity of diagnostic tests can be valuable during program evaluation to assess whether transmission has been interrupted by ensuring with reasonable certainty that there are no false negatives. A diagnostic test with lower sensitivity will increase the sample size required to estimate endpoints which increases resources needed from implementing programs (reviewed in [89]). However, maintaining high sensitivity may require the loss of specificity and necessitates careful assessment of cut-points to maximize both sensitivity and specificity for a given test.

Specificity refers to the proportion of truly negative individuals that had a negative test out of the total number of non-infected individuals (discussed in [88]). Characterizing the specificity of diagnostic tests allows programs to anticipate the number of individuals that may test positive but are not infected. Low specificity may not be a critical issue when implementing interventions, such as IVM MDA, that are not harmful if not-infected and the priority is high levels of coverage to interrupt transmission. Alternatively, as programs evaluate their progress towards elimination, maximizing specificity is particularly important. This is especially relevant when considering the WHO guidelines for confirming interruption of transmission that require a seroprevalence of less than 0.1% in children less than ten years-old [38]. Currently Ov16 is the accepted serologic marker of infection; however, even with reported estimates of specificity for Ov16 as high as 98% the number of false positives identified will exceed the number of false positives allowed by WHO guidelines [38, 62]. The guidelines require skin biopsies to be performed on all serologically positive children immediately and at a one and a half year follow up in the absence of ivermectin treatment of the children to allow for sufficient time for effects of IVM to no longer be a concern

[38]. This process is time intensive, and it is difficult to ensure that children are not lost to follow up. Adding a confirmatory test such as Ov33 to maximize the specificity could provide an alternative to skin snips. A second serologic test could easily be implemented given that the necessary blood samples were already collected, and it would provide immediate results for programs so they do not have to delay their verification of elimination.

Based on the individual probabilities for latent class assignment, we believe that the latent class was modeling patent infection versus non-infected. Thus, the estimated specificities for Ov16 and Ov33 using LCA model the ability of the diagnostic tests to accurately identify individuals not having patent infections, instead of as never infected. It is important to note that these estimates differ from earlier estimates reported in the literature for both Ov16 and Ov33. The variability in estimates of diagnostic accuracy depends on the populations they are being evaluated in. In a hyperendemic setting with limited IVM coverage, it would be expected that the estimated specificity would be different than estimates calculated using a non-endemic population. This explains in part the difference in LCA estimates of specificity for Ov16 compared with the reported specificity by Lobos and colleagues given that their estimates were based on evaluation two populations: individuals with confirmed parasitologic findings and individuals with no exposure to *O. volvulus* [62]. Additionally, the high specificity estimated by Lucius and colleagues for Ov33 was calculated similarly comparing a population of known onchocerciasis patients and a population of individuals with other known filarial infections [74].

Characterization of the combined specificity of Ov16 and Ov33 was one of the tentative aims of this study given the potential increase in specificity that we anticipated adding Ov33 to Ov16 would facilitate. The expected combined specificity of Ov16 and Ov33 is estimated to be 93.9% if individual specificities were directly multiplied. Given that both are filarial antigens cross-reactivity to similar filarial parasites makes it difficult to conclude that the assumption for independence between Ov16 and Ov33 was met, thus this estimate is not robust [62, 74]. We tried to use the latent class model to evaluate the four combinations of combined test results for Ov16 and Ov33 in an effort to generate estimates of combined specificity. It was not feasible to do this because we could not determine the proportion of individuals that would have tested positive or negative. The two tests were combined into four categories, but two of the categories included discordant test results and could not easily be assigned to a positive or negative group. We considered making assumptions about which group to assign the categories with discordant test results; however, determined that this was not a robust method of estimation.

Using LCA to evaluate the diagnostic accuracy of Ov16 and Ov33 was a useful exercise as it provided the opportunity to explore relatively new statistical methodology that had not been previously applied to evaluating diagnostic tests for onchocerciasis. Unfortunately, our analysis was limited by the statistical software and the assumptions of LCA. The latent class model was determined from a computer-based algorithm that does not allow for modifications of latent class assignment on an individual basis to reduce misclassification. For example, there were several parasitologically positive individuals that were not included in the latent class identified as infected; however, there were no

options to require that parasitologically positive individuals be included in the infected latent class. One of the benefits of using a three-class model was that it corrected for some of these inconsistencies by separating out individuals with conflicting serologic and parasitologic results into: patent, infected but patency unclear, and non-infected classes. Though the three-class model had an intuitively better fit, it could not be used to calculate estimates sensitivity and specificity. The inconsistencies between parasitologic and serologic tests can be explained in part by the decrease in mf density following IVM MDA as well as the inherent limited sensitivity of parasitologic methods.

This study is a preliminary analysis of the diagnostic accuracy of Ov16 and Ov33, and we recognize that there are a number of limitations that need to be addressed. This was a secondary analysis and was not powered to include clusters in regression analyses. However, the primary objective of the regression analysis was to identify associations between covariates and serologic tests and not prevalence. By including country in the model we were able to control for the observed differences in distribution of covariates and identify meaningful associations between covariates and Ov6 IgG4 ELISA positivity. It is important to note that study participants often over-reported symptoms (it appears that many participants may have hoped that they would receive additional medical care or resources by more reporting more symptoms), which biased these results to indicate a greater prevalence of onchocerciasis-related disease than the truth. In order to address this, self-reported covariates were not included in multivariable analysis if there were equivalent clinical data.

We recognize that the potential implications of this analysis could improve elimination efforts, and propose several next steps to strengthen the conclusions that can

be drawn about the combined diagnostic accuracy of Ov16 and Ov33. We will continue to explore the capacity of PROC LCA to generate estimates of sensitivity and specificity of Ov16 and Ov33 combined as a four-level categorical variable. Other software programs will be reviewed to determine if they can estimate combined sensitivity and specificity of two diagnostic tests. If the limitations of statistical software cannot be resolved, a new study may be conducted to specifically evaluate the combined specificity of Ov16 and Ov33 in the future.

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Tables

Table 1. Descriptive statistics for study population in Ethiopia and Uganda (N=991).

	Ethiopia (N=497)		Uganda (N=494)		Total (N=991)		Test for Significance[a]	P-value
Covariate	**N**	**%**	**N**	**%**	**N**	**%**		
Demographics								
Age in years[c,d]	45.0	(8-90)	32.0	(10-92)	39.5	(8-92)	70.1	<0.0001
Male sex[d]	275	55.3	194	39.3	469	47.3	26.3	<0.0001
Farmer	446	89.7	354	71.7	800	80.7	52.0	<0.0001
IVM ever taken[b,d]	432	86.9	446	90.3	878	88.6	3.6	0.06
IVM taken in last year[b,d]	231	46.5	421	85.2	652	65.8	169.5	<0.0001
Spends most of day near river[d]	236	47.5	239	48.4	475	47.9	0.0	0.87
Home near river[d]	69	13.9	246	50.1	315	31.9	148.7	<0.0001
Participant reported symptoms								
Itching in the past year[d]	320	64.4	392	79.4	712	71.9	27.7	<0.0001
Itching disturbs sleep[d, e]	259	80.9	349	89.0	608	85.4	136.7	<0.0001
Skin changes[f]	122	24.6	129	26.1	251	25.3	138.3	<0.0001
Leg swelling[d]	116	23.4	58	11.8	174	17.6	22.9	<0.0001
Changes in vision[d, g]	322	64.9	323	65.7	645	65.2	0.1	0.81
Eye examination								
Impaired visual acuity[h]	52	10.5	21	4.3	73	7.4	14.0	0.00
Corneal eye lesions[d]	18	3.6	7	1.4	25	2.5	4.9	0.03
MFAC[b,d]	2	0.4	27	5.5	29	2.9	22.5	<0.0001
Any pk[b]	18	3.6	14	2.9	32	3.3	0.5	0.49
Pk stages A & B[d,h,i]	3	0.6	1	0.2	4	0.4	--	0.62
Skin examination								
Presence of skin nodules[d]	315	63.5	201	40.9	516	52.1	50.4	<0.0001
Number of skin nodules[c]	1.0	(0,11)	0.0	(0,16)	1.0	(0,16)	49.1	<0.0001
DPM[b,d]	180	36.4	43	8.8	223	22.7	171.0	<0.0001
APOD[b,d]	140	28.3	10	2.0	150	15.3	131.4	<0.0001
CPOD[b,d]	124	25.1	32	6.5	156	15.9	63.4	<0.0001
ATR[b,d]	122	24.7	48	9.8	170	17.3	38.0	<0.0001
LOD[b,d]	47	9.5	27	5.5	74	7.5	5.6	0.02
Hanging Groin[d]	8	1.6	4	0.8	12	1.2	1.3	0.25
Lymphadenopathy[d]	117	23.5	0	0.0	117	11.8	131.5	<0.0001
Lymphedema[d, i]	73	14.8	2	0.4	75	7.6	--	<0.0001
Hydrocele (males only)[d]	4	1.5	12	6.3	16	3.4	7.8	0.01

Lab diagnostics								
Ov16 IgG4 ELISA positive[b]	360	72.4	414	83.8	774	78.1	18.7	<0.0001
Ov16 MBA positive[b]	379	76.3	421	85.2	800	80.7	12.8	0.0003
Ov33 MBA positive[b]	407	81.9	412	83.4	819	82.6	0.4	0.53
Ov17 MBA positive[b]	305	61.4	329	66.6	634	64.0	2.9	0.09
Skin snip microscopy positive	20	4.0	127	25.7	147	14.8	92.2	<0.0001
Average number mf per snip[b,c]	0.0	(0,180)	0.0	(0,105)	0.0	(0, 180)	278.3	<0.0001
Skin snip PCR positive [b,d]	59	11.9	150	30.4	209	21.1	50.7	<0.0001
Skin snip qPCR positive [b,d]	64	12.9	161	32.6	225	22.7	54.6	<0.0001
Rapid format ICT positive[b,d]	15	3.1	0	0.0	15	1.5	15.4	<0.0001

[a] Tests for significant difference between Ethiopia and Uganda included: chi-square test, Fisher's exact test, and median test.

[b] ivermectin (IVM); microfilaria in the anterior chamber of the eye (MFAC); microfilaria (mf); acute papular onchodermatitis (APOD); chronic papular dermatitis (CPOD); lichenified onchodermatitis (LOD); depigmentation (DPM); atrophy (ATR); punctate keratitis (pk); enzyme-linked immunosorbent assay (ELISA); multiplex bead assay (MBA); polymerase chain reaction (PCR); real-time polymerase chain reaction (qPCR); immunochromatograph card test (ICT)

[c] Reported as median and range.

[d] 1 missing for PCR and qPCR. 2 missing for sex. 3 missing for leg swelling. 4 missing for home near river, itching in past year, and nodules. 5 missing for corneal lesions and hydrocele. 6 missing for ivermectin ever taken, ivermectin taken last year, MFAC, any punctate keratitis, punctate keratitis stages A & B, and lymphedema. 8 missing for ATR, DOM, CPOD, LOD, APOD, lymphadenopathy, and hanging groin. 10 missing for spend most of day near river. 11 missing for age. 104 missing for itching disturbs sleep.

[e] Reported as percentage of individuals that reported that they experienced itching in the past year.

[f] Skin changes reported by participants included: depigmentation, sagging, increased wrinkling, and thinning of the skin.

[g] Vision changes reported by participants included: cannot see at all, blurred vision, can see light and dark only, cannot see well at night, and cannot distinguish faces and details.

[h] Impaired visual acuity was defined according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10), WHO criteria for visual impairment (visual acuity less than 20/200).

[i] Fisher's exact test reported.

Table 2. Bivariable analysis. Association between covariates and positive Ov16 IgG4 Enzyme-linked immunosorbent assay (ELISA) for *O. volvulus* infection in Uganda and Ethiopia study population (N=991) controlling for country.

Parameter	β Estimate	SE[a]	P-value	Point Estimate (OR)[a]	95% CI[a]
Demographics					
Age in years[d]	0.01	0.00	0.02	1.01	(1.00, 1.02)
Male sex[d]	0.17	0.16	<0.0001	1.19	(0.87, 1.62)
Spends most of day or lives near river[d]	-0.40	0.16	<0.0001	0.67	(0.49, 0.91)
Farmer[d]	0.77	0.19	<0.0001	2.16	(1.75, 3.16)
IVM ever taken[a]	0.27	0.23	0.25	1.31	(0.83, 2.08)
IVM taken in the last year[a]	0.28	0.17	0.10	1.33	(0.95, 1.87)
Participant reported symptoms					
Itching in the past year	0.24	0.17	0.15	1.27	(0.91, 1.78)
Itching disturbs sleep	0.01	0.18	0.94	1.01	(0.71, 1.46)
Reported changes in skin					
Depigmentation of the skin	-0.23	0.20	0.24	0.79	(0.54, 1.17)
Sagging of the skin	-0.35	0.48	0.46	0.70	(0.28, 1.80)
Increased wrinkling (not on face)	0.19	0.40	0.64	1.21	(0.55, 2.64)
Thinning of skin	1.04	0.53	0.05	2.83	(0.99, 8.06)
Reported changes in vision					
Cannot see at all	0.46	0.63	0.47	1.58	(0.46, 5.44)
Blurred vision (during the day)	0.28	0.16	0.07	1.33	(0.97, 1.81)
Can see light and dark only[d]	0.74	0.29	0.01	2.10	(1.18, 3.73)
Cannot see well at night	0.15	0.30	0.60	1.17	(0.65, 2.08)
Cannot distinguish faces and details	0.75	0.49	0.12	2.11	(0.81, 5.48)
Eye examination					
Impaired visual acuity[b]	0.24	0.30	0.43	1.27	(0.70, 2.31)
Corneal eye lesions	1.34	0.74	0.07	3.83	(0.89, 16.48)
MFAC[a]	0.24	0.55	0.66	1.27	(0.43, 3.75)
Any pk present[a]	0.47	0.50	0.35	1.59	(0.60, 4.22)
Pk stages A & B	-0.02	1.17	0.98	0.98	(0.10, 9.61)
Any onchocerciasis-related eye involvement	0.15	0.50	0.76	1.16	(0.44, 3.10)

Physical examination						
Skin nodules[d]	0.65	0.16	0.00	1.91	(1.40,	2.59)
Number of skin nodules[d]	0.18	0.06	0.00	1.20	(1.06,	1.35)
Any OSD[a,c, d]	0.40	0.16	0.01	1.49	(1.09,	2.03)
Skin nodules only (no OSD) [d]	0.39	0.19	0.04	1.47	(1.01,	2.14)
OSD only (no skin nodules) [d]	-0.50	0.20	0.01	0.61	(0.41,	0.90)
Skin nodules and OSD[d]	0.50	0.19	0.01	1.64	(1.14,	2.37)
Lymphadenopathy	0.03	0.24	0.89	1.03	(0.65,	1.66)

[a] Standard error (SE); Odds Ratio (OR); Confidence interval (CI); microfilaria in the anterior chamber of the eye (MFAC); punctate keratitis (pk); onchocercal skin disease (OSD)

[b]Impaired visual acuity was defined according to the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10), WHO criteria for visual impairment (visual acuity less than 20/200).

[c]Onchocercal skin disease (OSD) includes the presence of any of the following: acute papular onchodermatitis (APOD), chronic papular dermatitis (CPOD), lichenified onchodermatitis (LOD), depigmentation (DPM), atrophy (ATR), and hanging groin.

[d]The following covariates were significant at the alpha = 0.05 level: age in years, male sex, spends most of day or lives near river, farmer, can see light and dark only, skin nodules, number of skin nodules, any OSD, skin nodules only (no OSD), OSD only (no skin nodules), and skin nodules and OSD.

Table 3. Multivariable analysis. Association between covariates and positive Ov16 IgG4 Enzyme-linked immunosorbent assay (ELISA) for *O. volvulus* infection in Uganda and Ethiopia study population (N=991) controlling for country.

Parameter	β Estimate	SE[a]	P-value	Point Estimate (OR)[a]	95% CI[a]
Age in years	0.00	0.01	0.51	1.00	(0.99, 1.01)
Male sex	0.13	0.16	0.44	1.14	(0.82, 1.56)
Farmer	0.44	0.22	0.04	1.55	(1.01 2.39)
Skin nodules at physical exam	0.78	0.17	<0.001	2.17	(1.55, 3.04)

[a]Standard error (SE); Odds Ratio (OR); Confidence interval (CI).

Table 4. Comparison of sensitivity and specificity estimates for Ov16 IgG4 Enzyme-linked immunosorbent assay (ELISA) and Ov33 multiplex bead assay (MBA) for *O. volvulus* infection using skin snip results and latent class analysis (LCA) estimates in Ethiopia and Uganda study population (N=991).

	Ov16 IgG4 ELISA		Ov33 MBA	
Measure	**Sensitivity**	**Specificity**	**Sensitivity**	**Specificity**
Skin snip results[a]	92.7%	26.3%	94.0%	20.9%
Latent class analysis[b]	93.7%	79.4%	96.9%	70.4%

[a] Positive skin snip microscopy and/or real-time polymerase chain reaction (qPCR) result were considered true positives.
[b] Latent class analysis model included skin snip microscopy, qPCR, Ov16 IgG4 ELISA, Ov33 MBA, and Ov17 MBA to determine class assignment.

Chapter III. Public Health Implications

1. The primary objective of this study was to evaluate the currently available diagnostic tools with the goal of identifying a way to detect onchocerciasis cases with greater specificity for the elimination program surveillance. This study provides robust estimates of sensitivity and specificity for two diagnostic tests—Ov16 and Ov33—and demonstrates that these estimates are much higher compared with sensitivity and specificity calculated using a 2x2 contingency table with parasitologic results as truth. Our results demonstrate that serologic tests are much better at identifying patent infections than parasitologic methods would suggest given that there were significantly fewer false positives than the 2x2 calculation predicted. This is valuable for programs monitoring their progress towards elimination.
2. Our results provide evidence for the potential use of Ov33 as confirmatory test. The availability of a second diagnostic serologic test could simplify confirmatory testing of individuals that have positive Ov16 results by allowing for a faster, less invasive alternative to the skin biopsies. Endemic countries in Sub-Saharan Africa that are in the monitoring and evaluation phase of their elimination programs need diagnostic methods that are sufficiently specific to meet the WHO threshold for elimination of transmission of onchocerciasis in humans.
3. The use of LCA in this analysis has highlighted some of the significant limitations of this statistical methodology. Future analyses using LCA to estimate the diagnostic accuracy of tests in the absence of a gold standard should be wary of drawing conclusions that the methodology is not robust enough to support.