Diagnostics for onchocerciasis in the era of elimination

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In the past few years, efforts to eliminate onchocerciasis from Africa have intensified. These efforts are primarily based on the mass distribution of the anti-helminthic drug Mectizan™ (ivermectin). This program has led to the development of new guidelines by the World Health Organization for the verification that transmission has been suppressed and eventually eliminated. The requirements of diagnostic tools for this purpose differ in many ways from tests used to diagnose infection in individuals. In this review, we summarize the progress that has been made to identify diagnostics that meet the specialized requirements needed to verify onchocerciasis elimination, discuss why these tests were selected and summarize the needs that still exist to complete the arsenal of diagnostic tools that will be useful as the goal of elimination is achieved.

Introduction

A recent review has put forth the challenges faced by onchocerciasis elimination programs and how their diagnostic needs evolve as they transition from control verification of elimination.[1] Here, we will focus upon the diagnostic needs to verify suppression and interruption of transmission in the context of the current WHO guidelines.

Currently, onchocerciasis elimination programs rely primarily on mass drug administration (MDA) of Mectizan™ (ivermectin) to suppress and eventually eliminate transmission of *Onchocerca volvulus*, the causative agent of the disease. The onchocerciasis elimination program strategy is a multi-stage process.[2] Initially, programs must obtain high enough treatment coverage in the eligible population to stop transmission. Once transmission is suppressed, high coverage must be maintained until all the fertile female parasites either die or become sterile. Once a suitable number of treatments have been given, transmission is believed to have been interrupted. At this point in time, the program conducts a comprehensive survey to demonstrate that transmission has been interrupted. These surveys rely upon measuring parasite exposure in children under 10, who should have remained naive to exposure had transmission been suppressed and whose test results represent a surrogate measure of exposure incidence in the population. The current WHO guidelines state that the upper bound of the 95% confidence interval (CI) for the exposure prevalence in the population of children under 10 cannot exceed 0.1%.[2]

In addition to the measurement of the prevalence of exposure in children, the current guidelines also call for an entomological evaluation to be conducted.[2] In this evaluation, the upper bound of the 95% CI of the prevalence of flies carrying *O. volvulus* infective larvae must be less than 0.05%.[2] If both the epidemiological and entomological criteria are met, it may be concluded that transmission has been interrupted. At this point MDA activities can be discontinued, and the program enters the surveillance phase of the elimination process. Three to five years after treatments have been discontinued, the program conducts another entomological evaluation, where once again, the upper bound of the 95% CI of the prevalence of flies carrying *O. volvulus* infective larvae must be less than 0.05%.[2] If this criterion is met, it can be concluded that onchocerciasis has been eliminated in the evaluation area.

The capability of a diagnostic test is challenged in situations where one is trying to prove that something no longer exists. A test's sensitivity is the probability of a positive test in a population that is infected, while specificity is the probability of a negative test in an uninfected population. Adjusting test cut-offs to improve one parameter has the opposite effect on the other parameter (e.g., increasing sensitivity will result in a decrease in specificity). Two measures that reflect the accuracy of a test are the positive and negative predictive values. The positive predictive value is the

proportion of true positives in the overall number of positives reported by the test, while the negative predictive value is the proportion of true negatives in the overall number of negatives reported. These measures are driven by the sensitivity and specificity of the test and by the infection prevalence in the population. In the setting of a disease elimination program, prevalence is near to or at zero. In the absence of a highly specific test, most positive results will be false positives, and the positive predictive value of the test will be very low.

Because the elimination strategy requires that all eligible individuals in a targeted community regardless of infection status be treated, the results of any particular test will not affect an individual's treatment. Thus, test sensitivity can be sacrificed to maximize specificity and minimize the number of false positive results in a situation where one is interested in population prevalence, as the number of people sampled can be increased to compensate for decreases in sensitivity.[3] For example, as discussed above, the epidemiological criterion for stopping MDA in the current WHO guidelines for verifying interruption of transmission of O. volvulus requires testing enough children to conclude that the upper bound of the 95% CI of the prevalence of exposure in at-risk children less than 10 years old is less than 0.1%.[2] Assuming a test with 100% sensitivity and 100% specificity, 3000 children must be tested and have negative results to meet this criterion.[4] The sample size needed for a test exhibiting less than 100% sensitivity is roughly the sample size needed for an assay with 100% sensitivity divided by actual sensitivity.[3] For example, for an assay with 70% sensitivity, one needs to test approximately 4285 individuals to meet the WHO criterion. In contrast to sensitivity, the specificity of an assay sets a floor of a prevalence value below which it is very difficult to measure. For example, if we employ an assay with a specificity of 99%, roughly 1% of the samples will test falsely positive in every trial. This means that the test on average will report a prevalence rate of 1%, even if the true prevalence is 0%. To detect a true prevalence of 0.1% in this case, sufficient individuals must be tested to ensure that a result reporting a 1.1% prevalence is significantly different from the 1% false positive rate. To achieve this goal would require testing over 63 000 individuals (C. R. Katholi, personal communication). Thus, ensuring a very high degree of specificity in the assay used to verify transmission elimination is paramount.

Detection of parasite presence in humans

Traditionally, microscopic examination of skin biopsies (snips) has been the gold standard for diagnosis and surveillance of O. volvulus infection.[5] Snips generally exhibit a high degree of specificity, as O. volvulus and Mansonella streptocerca are the only filarial parasites in onchocerciasis endemic areas whose larvae inhabit the skin of an infected individual, and the larvae of these two species are readily distinguishable. However, snips are generally insensitive indicators of infection and the sensitivity of the skin snip decreases as the density of microfilaria in the skin decreases.[6] This problem is exacerbated in populations that have received MDA with Mectizan™, which is a potent microfilaricide that effectively reduces microfilarial density in communities under successful MDA. A recent report suggests that the sensitivity of the conventional skin snip assay when compared with PCR in areas subject to successful MDA ranged from 76% in Uganda to 29% in Ethiopia.[6]

Attempts have been made to increase the sensitivity of the skin snip assay by replacing the microscopic examination of the snip with detection of amplified parasite DNA. The original DNA amplification assay for O. volvulus targeted a tandemly repeated sequence present in the O. volvulus genome with a unit length of roughly 150 bp, designated the O-150 repeat.[7] This repeat family was found to be present in other species of the genus Onchocerca, but was lacking in the other human filarial parasites.[7] The repeat family consisted of genus, species and strain-specific repeat units,[8] permitting the development of species and strain-specific probes that could be used to classify the amplicons generated from amplification of the O-150 repeat.[9] Real-time PCR[6,10] and isothermal loop amplification (LAMP)[11,12] assays have also been developed for the amplification of O. volvulus DNA, decreasing the limit of detection of these assays to significantly less than a single parasite and permitting rapid colorimetric detection of the amplified products.[13] Most of these assays have targeted the O-150 repeat, though similar assays have been reported that target moderately repeated DNA sequences (rRNA genes,[6] mitochondrial genes[11]) or even single copy genes.[13] As might be predicted, using a DNA amplification assay to detect the presence of parasite DNA rather than using microscopy to detect the parasite itself has generally been found to increase the sensitivity of the skin snip.[6,10,14] As a result, amplification of O. volvulus parasite DNA from skin snips has become the accepted standard for the diagnosis of patent O. volvulus infection in humans.[2]

Despite the high specificity exhibited by the skin snip assays, these are not generally applicable for demonstration of transmission interruption for several reasons. First, Mectizan™ rapidly reduces the number of microfilariae in the skin to zero or near zero,[15] reducing the positive predictive value of the assay. Second, obtaining the biopsies is both painful and carries some risk of transmitting blood-borne infections, leading to community resistance.[16] Together, these drawbacks led WHO to recommend against the use of skin snip-based assays as a primary diagnostic for the verification of elimination.[2]

An alternative method to skin snipping is the diethylcarbamazine (DEC) patch test. This test is based upon the observation that application of the anti-helminthic DEC to the skin of microfiladermic individuals infected with O. volvulus elicits a localized rash within 24–48 h.[17] This is less invasive than collecting skin biopsies and thus potentially more acceptable to communities. DEC patch performance has varied widely in the different trials, with sensitivities ranging from 36 to 83% depending upon the comparator test (skin snip microscopy or PCR) used as the gold standard.[18–20] Specificities were generally not high enough for the DEC patch to be used as a stand-alone test in areas with low prevalence.[19] Furthermore, the sensitivity and specificity of this test was not evaluated in situations where successful MDA programs were ongoing. These issues have prevented the DEC patch test from being recommended by WHO for the verification of elimination.[2]

Serological tests to detect exposure to O. volvulus

Preliminary studies employing low molecular weight (LMW) O. volvulus protein fractions as antigens in serological assays resulted in promising levels of sensitivity and specificity.[21,22] As a result, multiple LMW antigens were produced and evaluated for the serodiagnosis of onchocerciasis (Table 1).

Table 1. Candidate antigens considered for diagnosis of onchocerciasis

MW (kDa)	Antigen(s)	Associated protein	Specificity (%)	Sensitivity (%)	Reference	Test used
15	OV103	MF surface associated protein	70	57	23	ELISA
	Ov-MSA-1		99	89	24	IgG4 LIPS assay
16	OV16	Phosphatidyl ethanolamine binding protein	96	96	25	ELISA
17	OV10	Cysteine proteinase inhibitor	100	61	26	ELISA
	OC 9.3		83	68	26	ELISA
	OC 9.3		100	84	27	ELISA
	OV-CPI		99	32	24	IgG4 LIPS assay
	OV7		n/a	75	28	ELISA
19–20	OvMPB/10	Not determined	100	78	29	ELISA
20	OV11	Retinol binding protein	96	54	26	ELISA
	OvMPB/11		99	65	29	ELISA
	Ov-Far-1		100	100	24	IgG4 LIPS assay
	OV20/36M		100	45	30	ELISA
	OV20/OVS1		81/85	75/89	31	ELISA
20–23	OV 31	Not determined	92	68	26	ELISA
	OV 31			100	30	Microplaque spot analysis
	OV22/31M		100	74	30	ELISA
28	MSP-2	Major sperm protein	85	100	32	Dot blot assay
33	OC 3.6	Aspartyl protease inhibitor	n/a	93	27	ELISA
	OV33-GST		96	93	33	ELISA
	C27		n/a	82	34	Recombinant OV33/ELISA
	C71		n/a	85	34	Recombinant OV33/ELISA
	Ov-API-1		100	100	24	IgG4 LIPS assay
	OV 33/5M		100	n/a	30	ELISA

Currently, the field has settled upon assays detecting antibodies against the Ov16 antigen for monitoring exposure to *O. volvulus*. This antigen is present in all lifecycle stages[35] and elicits detectable antibody responses prior to the appearance of microfilaria in some chimpanzees experimentally infected with *O. volvulus*[36] and in some children exposed to the parasite in endemic communities.[37] While the initial assessment of the utility of the Ov16 antigen was assessed using detection of total IgG against Ov16, the IgG4 subtype response was the most specific.[38,39] This is perhaps not surprising, as measurements of IgG isotypes in filarial infections revealed that IgG4 accounts for up to 95% of the IgG response to these infections.[40] All current versions of the Ov16 assay have focused on IgG4 detection; however, the IgG4 response takes time to develop[41] and thus will not immediately reflect exposure to *O. volvulus*. The Ov16 ELISA is now recommended by WHO guidelines for demonstrating the interruption of transmission of *O. volvulus*.[2] Most Ov16 ELISA methods utilize dried blood spots (DBSs) as the input sample type. DBS samples are relatively stable, and can be easily collected and transported to a central facility for testing later. The cost of the reagents alone required for processing a single sample (generally run in duplicate) are approximately US $0.30, and one individual can process roughly 15 000 samples per year. However, this estimate does not include the cost of shipping to endemic laboratories. Fully burdened costs for running Ov16 ELISA are thus location-specific and should be analyzed as part of efforts to improve laboratory capacity in countries utilizing serological surveillance.

The Ov16 antigen was adapted into a rapid format card test by AMRAD ICT (Australia) with a reported sensitivity of 90.6%.[42] Despite promising field performance, production of this card test ceased in the year 2000. Recently, the interest in an Ov16 rapid test was revived, spurring the development of two rapid diagnostic tests (RDTs) incorporating the Ov16 antigen which are now commercially available. These consist of a single IgG4 rapid test and a combination test utilizing Ov16 and the *W. bancrofti* antigen Wb123.[43,44] The product inserts report the sensitivity of the Ov16 single test to be 81.1% and the Ov16 test line of the biplex test to be 81.33%, respectively.[45] The specificity is listed at 99.0% for the single Ov16 test and 100% for the biplex test, although it was reported to be 1–2% lower than these values in published studies using early prototypes.[43,44] As part of the development of the RDT, a recombinant human IgG4 positive control antibody specific for Ov16 was also developed,[46] providing a highly pure, consistent and long-term source of positive control for both the ELISA and RDT assays.

The new Ov16-containing RDTs for anti-Ov16 serology are rapidly being incorporated into field studies and surveillance activities. While feasibility and acceptability of the rapid tests in surveillance have been demonstrated,[47] field-based studies that include performance data using the commercial product are still pending publication. Newly released WHO guidelines recommend evaluation of the performance of these RDTs prior to their use in stop-MDA assessments;[2] hopefully, data to assess the utility of the Ov16 RDT in the verification of elimination will be available soon.

Entomological surveillance of *O. volvulus* transmission

The transmission cycle of *O. volvulus* includes blackfly vectors of the genus *Simulium*. The most direct measure of the status of transmission is to measure infectivity in the black fly vector population itself. Entomological surveillance has the advantage that it eliminates the time lag inherent in the assays focusing on the human host population, where detectable patency lags infection by 12–18 months.[37] The disadvantage to entomological surveillance is that large numbers of vector insects must be caught and tested. The current WHO guidelines call for testing sufficient numbers of flies to ensure that the upper bound of the 95% CI of the prevalence of flies carrying infective larvae is less than 0.05% (1/2000).[2] Meeting this criterion requires testing at least 6000 flies and having all test negative to meet this criterion.[4] Annual transmission potential can be used as an alternative criterion when it is not possible to capture 6000 flies due to low prevalence of flies.[2]

The traditional method of determining the prevalence of flies carrying infective larvae has been through field dissection of captured flies. However, this method suffers from two disadvantages. First, dissection is expensive because it requires a trained entomologist, a field microscope and a lot of time. Second, a more significant disadvantage is that *Simulium damnosum* sensu *lato*, the major vector of *O. volvulus* in Africa, also serves as the vector for zoonotic species of *Onchocerca* that do not infect humans.[48] The larvae of these animal parasites are difficult or impossible to distinguish morphologically from *O. volvulus*; thus, dissection data can result in overestimates of the intensity of transmission.

A solution to the inability of vector dissection to accurately describe transmission was to develop a specific PCR, and techniques to overcome the cost and time implications of having to test 6000 blackflies individually. Although several DNA amplification assays have been developed, the O-150 PCR has been the assay that has been used most widely.[13,49] This PCR method distinguishes *O. volvulus* from other Onchocerca present in *S. damnosum* s.l., thereby improving the accuracy of the transmission estimates.[9] Screening efficiency using the PCR is superior to dissection, as the PCR assay can be applied to screening pools of flies.[49] However, one potential drawback to screening pools of flies is that the O-150 PCR is not quantitative and inhibitors present in the DNA preparations can reduce the efficiency of the PCR, making it impossible to get accurate estimates of the number of parasites present in a positive pool, even when the O-150 PCR is adapted to a quantitative PCR format. Thus, it is not possible to determine whether a positive pool contains a single infectious fly or multiple infectious flies. This problem was overcome by applying probability distribution estimates when it was realized that although it was not possible to determine how many positive flies were in a positive pool, it was possible to say with certainty that negative pools contain no infectious flies. If the infectious flies are randomly distributed among the collection (something easily accomplished when arranging the flies into pools with the maximum number of flies appropriate for processing), it is possible to use a probability distribution to calculate the probability estimate of the number of infected flies in a pool, given the proportion of negative and positive pools and the number of flies contained in each pool.[49] The mathematics behind this observation were incorporated into a program (PoolScreen) that calculates the prevalence of infectious flies and associated confidence intervals from the proportion of positive pools, the pool size and the number of pools screened. Field studies conducted in both Africa[50] and Latin America[51] validated this approach. The O-150 PCR has subsequently been widely applied to collect entomological data verifying elimination of transmission of *O. volvulus* in Mexico,[52] Guatemala[53] and one focus in Sudan.[54]

PCR pool-screening techniques have overcome most of the operational difficulties associated with meeting the WHO guideline's vector criterion. Screening the 6000 flies necessary to meet this requirement by screening just 60 pools of 100 flies each would take one individual less than 1 week. Pool screening also dramatically reduces the cost and time necessary to process the samples, when compared to analyzing each insect individually. The cost of reagents to process a single pool of 100 flies roughly US$6.90 per pool, or roughly US$ 0.07 per insect. A single individual can process roughly 4000 pools or 400 000 individual insects in a year. Furthermore, the collected insects can be stored in alcohol indefinitely at room temperature, minimizing the logistical difficulties encountered when shipping the collections to a central laboratory for analysis.

Collecting the necessary number of flies now represents the main challenge to implementing the entomological surveys. Currently, the standard method of collecting vector black flies is human landing collections (HLCs), which are quite inefficient, as a team of two collectors can only collect one person-day's worth of flies per day. However, recent reports suggest that a new trap platform, known as the Esperanza Window Trap (EWT), may represent an effective alternative to HLCs for collecting vector black flies.[55] Studies in Mexico demonstrated that EWTs operated by residents of the affected communities could collect sufficient numbers of flies to certify these communities were free of *O. volvulus* transmission.[56] If the EWT platform has an equivalent performance in Africa when operated by community members, it may overcome the difficulty in obtaining sufficient numbers of vector flies to demonstrate suppression and interruption of transmission.

Conclusion and future directions

Although the current diagnostic tools have served well for verifying suppression and interruption of transmission of *O. volvulus* in most countries in Latin America and in several foci in Africa, there are several tools that could accelerate program activities targeting the elimination of onchocerciasis. One of the most pressing needs is to define the sampling schemes for verification of elimination in Africa. In Latin America, the approach that was used by the Onchocerciasis Elimination Program for the Americas was to identify sentinel hyper-endemic communities in each focus prior to the beginning of the program.[57] The epidemiological and entomological indicators described previously were then used to follow the decline and eventual elimination of transmission in these sentinels. In general, the foci in Latin America were also isolated from one another, making the definition of a focus a simple matter. In contrast, sentinel communities were often not identified prior to the start of onchocerciasis control in Africa. Furthermore, the foci that exist in Africa

are often not isolated, with the potential for reintroduction of the parasite either through migration of infected people or by wind-borne flies.[58] It is thus necessary to gain a better understanding of what the limits to a focus are and, once this is done, how these foci should be sampled. For example, the epidemiological metrics needed for stopping MDA still need to be clarified, including how many communities should be enrolled to obtain reliable data on transmission in a focus, how many people in each community should be sampled and how the communities to be included should be chosen. Similarly, for the entomological metrics, it is not clear how many different sites should be included, how many flies need to be collected from each site, or for how long and how often the collections should be carried out.

A second need is for the current seroprevalence cut-offs in the 2016 WHO guidelines to be re-evaluated in light of the recent progress in modeling the transmission of *O. volvulus*. For example, a recent study has suggested that the prevalence of exposure in children that would indicate the parasite population is irreversibly headed to extinction may often be higher than 0.1%, though this is dependent on the baseline endemicity.[59] A similar re-evaluation of the entomological metrics is also in order, perhaps with more focus on the annual transmission potential, rather than simple prevalence of infection. Once these analyses are completed, they should be used in conjunction with methods to both more accurately determine test performance in relevant settings and calculate sample sizes that take the sensitivity and specificity of the tests to be employed into account.[3]

Specificity is paramount when choosing tests for verifying elimination. However, a single-antigen antibody test often cannot deliver a very high degree of specificity without suffering a dramatic loss of sensitivity. One solution is to use a confirmatory test that is independent from the primary test, and require that both the primary and confirmatory tests be positive before declaring a sample positive. Incorporating a second parasite antigen marker in an RDT, with a distinct line for each marker, could allow tailoring of the test to provide either highest sensitivity (requirement of only a single test line present to be positive) or highest specificity (both test lines must be positive). In a similar vein, while the sensitivity and specificity of the PCR assays used in black flies generally approach 100%, a major technical obstacle with these assays is the potential for amplicon contamination resulting in false positive signals. Including an independent PCR assay targeting a second genomic sequence is one way to overcome this problem. Given that there are other antigens already available that can be used to detect exposure to *O. volvulus*, and other PCR targets already identified for detection of parasite DNA, studies and necessary product development should be undertaken to determine which combination of tests would result in the highest combined sensitivity and specificity for verifying elimination.

Finally, the available diagnostics do not directly detect potentially fertile female parasites in the human population that could restart reproduction once MDA is stopped and which may pose a risk for recrudescence. Even though not all adult worms need to be sterile or dead for transmission to be irreversibly interrupted, it would be beneficial to have a method to detect and treat people who harbor adult worm infections. The detection of adult worm infections could allow targeted treatment with medications that can permanently sterilize or kill the worms, either to accelerate program progress to achieving interruption of transmission or to further lower the probability of recrudescence of transmission in mop-up operations that occur after MDA has been stopped but transient transmission is detected. Safe treatments are available that can permanently sterilize and eventually kill the adult females,[60] so the missing piece is the diagnostic test to identify people infected with fecund adult females. Some progress has been made in developing assays to detect viable adult parasites in humans. These include specific metabolites produced by female worms[61,62] and detection of parasite miRNA in the blood of infected individuals.[63] Assays that could detect patent infections in the face of an effective MDA program have great potential for both speeding up the process of elimination and ensuring that the infection does not recrudesce once MDA is withdrawn.

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