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Diagnostic Challenges in Monitoring and Evaluating Annual Ivermectin (Mectizan®) Treatment Intervention Strategy to Determine Onchocerciasis Elimination Status

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Summary

Ability to accurately detect an active infection depends largely on the high accuracy (sensitivity and specificity) of a diagnostic test. There is no single diagnostic tool that meets the criteria of been simple, measurable, accurate, repeatable and timely (SMART). Combining the screening and confirmation tests have been relied upon in determining the true epidemiological situation of ongoing ivermectin or Mectizan® treatment intervention strategy. Antibody detection assays have been evaluated as screening tools using various formats like different types of agglutination protocol, enzyme linked immunosorbent assays (ELISA) and nucleic acid amplification tests (NAAT). Despite these developments, presence of any stage of the parasite antigen, microfilaria (fourth larva, L4) and macrofilaria (adult worm) confirms active infection. The standard microscopic examination of skin snips for emerged microfilaria obtained with a scleral biopsy punch is capable of spreading blood viral diseases. The sensitivity of the test has decreased following post-treatment with ivermectin and surveillance for recrudescence is inevitable. On one hand, the time preceding when parasite materials are liberated as against when detectable immune responses were invoked decreases the sensitivity of serological tests. The DNA molecular based tests with the attendant complexity of protocol remained a technical barrier that has been surmounted with the development of loop-mediated isothermal amplification (LAMP). This technique amplifies DNA with high specificity, sensitivity and rapidity under isothermal conditions. It will serve as alternative definitive diagnostic test to skin microfilaria detection. A combination of LAMP with IgG4 antibodies against *O. volvulus* 16 kiloDalton (Ov16) antigen is used as a rapid qualitative test of human finger-prick blood, serum and plasma now widely applied in disease endemic countries. Also, diethyl carbamazine (DEC) patch skin test for popular eruption indicative of positive infection attracted further re-evaluation as non-invasive screening tool devoid of skin biopsy and blood sample collection.

Keywords: Diagnosis, Microfilaria, Onchocerciasis, Post-treatment, Screening-tests, Surveillance.

Introduction

The onchocerciasis or river blindness is transmitted through the bite of the insect vector, a ferocious blood sucking blackfly, *Simulium damnosum sensu lato* Theobald. When about 2-3 microfilariae are ingested they undergo developmental changes from first stage (L_1) to third stage infective larvae (L_3) in the blackfly. The classical skin snip test (Figure 1) for emerged microfilariae (mf) referred to as the "gold standard" is invasive and less sensitive. Its sensitivity depends on location or site, number of skin snips taken and level of skin mf density (Taylor *et al*., 1989). It is expected to become further less sensitive due to reduction in microfilaria load at the period following post-treatment when surveillance (PTS) for recrudescence will become inevitable (Guzman *et al*., 2002). The advantage of antibody over antigen and nucleic acid detection is the ability to

detect past exposure to infection and current (active) infections (Enwenzor *et al*., 2000). Antibody test can provide the basis to define those to be included for diagnostic and or confirmation tests. The Yaoundé Ministerial Declaration emphasized the need for a sustainable and integrated approach for surveillance and control within strengthened health systems (Amazigo and Boatin, 2006). Economic consideration for lack of market has constrained investment in the development of diagnostics for NTDs. Another veritable test available is the patch skin test developed by Hutchison *et al*. (1979) and Stingl *et al*. (1984) is shown on Figure 2. Application of diethylcarbamazine (DEC) as a "patch skin test" (PST) to the skin of 45 patients with onchocerciasis in the Southern Sudan proved to be non-invasive with 69 (92%) were positive. A positive reaction was indicated by a papular eruption beneath the patch. The potential field application of DEC-PST for monitoring impact CDTI strategy have been evaluated by Laurent *et al.* (2000); and found to be very useful, simple, and inexpensive.

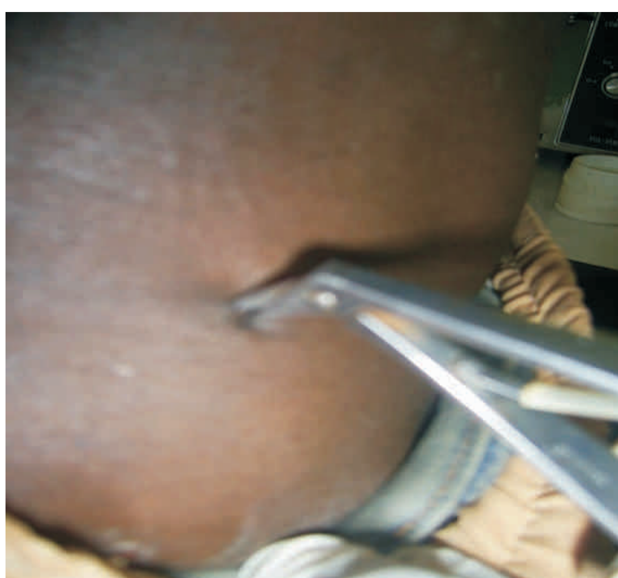

Figure 1: Skin snip using scleral biopsy punch

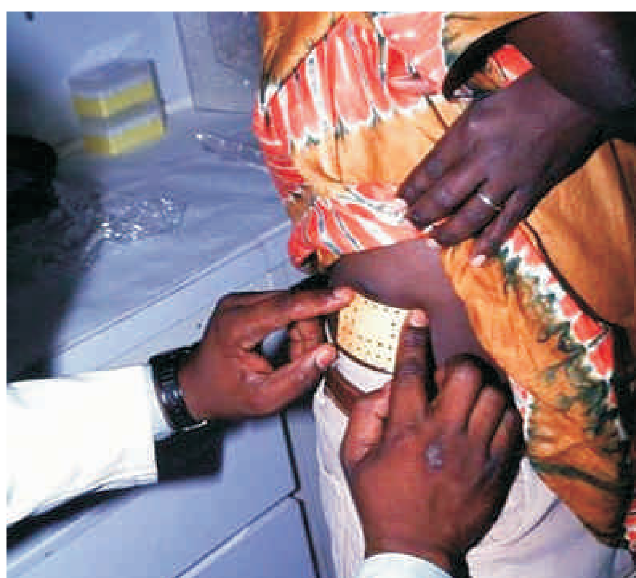

Figure 2: Patch skin test

A reliable diagnostic test to complement and overcome continued reliance solely on skin microfilaria detection regarded as the 'gold standard' has made the need for standard or quantitative PCR highly desirable. The two tests require specialized skill, costly equipment and rely on invasive skin snipping (Figure 3) which is not suitable for population surveillance. Ethical consideration due to risk of transmitting blood borne diseases such as human immunodeficency virus (HIV) and hepatitis B virus will depend on screening test that will limit the number of participants to undergo diagnostic test involving skin snipping. A diagnostic algorithm with screening, diagnosis and confirmatory tests will ensure effective and efficient surveillance and monitoring of onchocerciasis control programme. The screening test should be simple, measurable, accurate, repeatable and timely (SMART) as described by Mabey *et al.* (2004). Antibody detection assays meet the above criteria for a screening test that can readily be applied in the field. A novel onchocerciasis slide agglutination test (Oncho-SAT) is evaluated with samples from disease endemic communities. According to Mabey *et al.* (2004) and WHO (2005), the ideal test should be SMART, adaptable for field use and affordable by poor disease endemic countries (DEC).

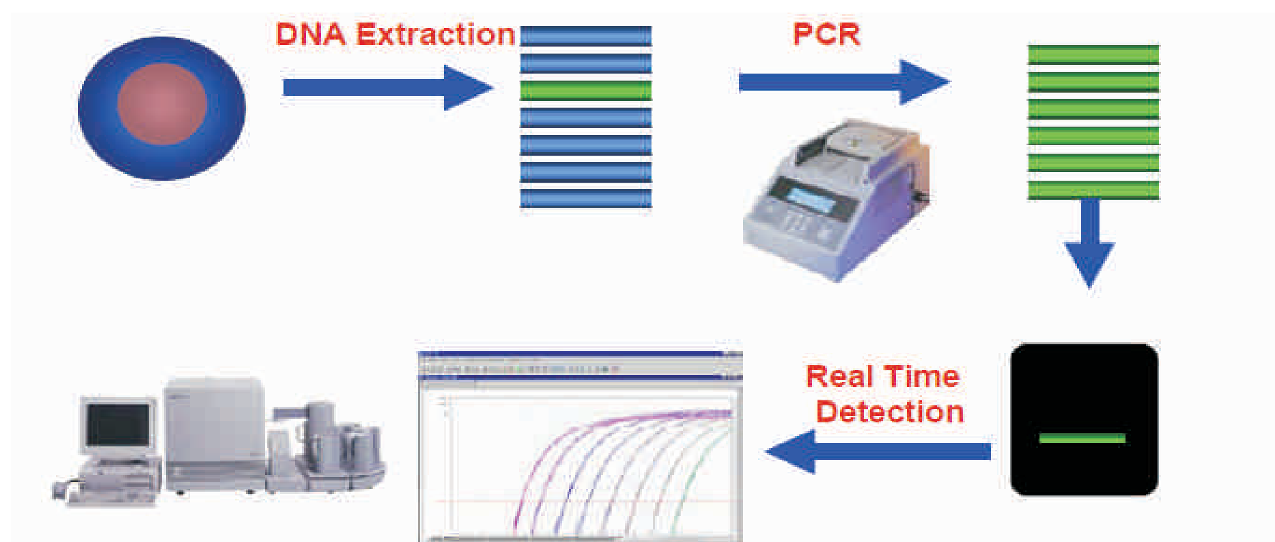

Figure 3: Real-Time versus Traditional PCR (Real-Time chemistries allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional methods use Agarose gels for detection of PCR amplification at the final phase or end-point of the PCR reaction).

Immunodiagnostic tests: Concerted efforts aimed at developing a definite serodiagnostic test was approached through dot blot immunobinding assay (DIA-BA) based on the biotin-avidin binding system, for the detection of *O. volvulus* specific antigens in body fluids. Comparing the antigen detection assays in urine, dermal fluid and tear specimen over sera that gave 100% sensitivity in skin mf positive subjects (Wembe *et al*., 2005). The biotinylated probes were then used to detect *O. volvulus* specific antigens initially blotted onto a nitrocellulose membrane. The smallest amount of blotted antigens detectable by the new test was 0.5ng, 1ng, 1ng and 2ng respectively in urine, dermal fluid, tears and serum samples. Similarly, an oncho-dipstick has been developed and evaluated, and was found capable of detecting up to 25ng antigen in urine and tears by Ayong *et al.* (2005). Sensitivity of the oncho-dipstick assay was 100% in urine and 92% in tears; its specificity was 100% in both. Concordance between urine and tear test results from the same individuals was 87%. Serodiagnosis has continued to attract more attention as a viable screening option (Lipner *et al*., 2006) that complements the less sensitive classical onchocerciasis diagnostic method. Comparative performance different screening test developed thus far are shown on Table 1.

The time preceding when parasite materials are liberated as against when detectable immune responses were invoked decreases the sensitivity of the serological tests. Detection of earlier or pre-patent infection particularly in children was facilitated with antibody serology as previously reported (Gbakina *et al*., 1992; Ogunrinade *et al.,* 1992; Ogunrinade *et al.,* 1993; Enwenzor *et al.*, 2000). However, a major setback to antigen detection is the need to subject the samples (serum or urine) to one form of pre-treatment or the other (More and Copeman, 1991; Mbacham *et al*., 1992). While serum -is treated to remove interference by specific host antibodies, urine is dialyzed to obtain concentrated residue. Hence, this will make the field application of antigen detection somehow a difficult process to accomplish. Recently, four recombinant *Onchocerca volvulus* antigens (Ov-FAR-1, Ov-API-1, Ov-MSA-1 and Ov-CPI-1) were tested by luciferase immuno-precipitation system (LIPS). This allowed for unequivocal differentiation between Ov-infected and uninfected control sera with 100% sensitivity and 100% specificity (Burbelo *et al*., 2009). The mixture of the 4 Ov antigens simultaneously in the standard format or a quick 15-minute format (QLIPS) showed 100% sensitivity and 100% specificity in distinguishing the Ov-infected sera from the uninfected control sera.

Table 1: Onchocerciasis screening and diagnostic tests developed

Study, diagnostic test	Sensitivity, %	Specificity, %	Remarks
Ayong *et al*. (2005): Urine antigen dipstick assay	100	100	Not yet
Tear antigen dipstick assay	92	100	available
Vincent *et al*. (2000): Serum antigen immunoblot	100[a]	100[a]	---
Skin-snip PCR ELISA	90[a]	100[a]	---
Serum IgG4 (OC3.6gst) ELISA	78[a]	100[a]	---
Serum IgG4 (OC3.6gst and OC9.3gst) ELISA	97[a]	100[a]	---
Zhang *et al*. (2000) Skin-snip PCR PCHA	88	100	---
Skin-snip PCR AGE	81	100	---
Skin-snip PCR ELISA	94	100	---
Weil *et al*. (2000):	91[a]	93-100[a]	---
Guzman *et al* . (2002): serum antibody dot blot assay	99	90	Field-use friendly
Nde *et al* . (2002): serum antibody ELISA (hybrid recombinant antigen OVH2)	98.5	95.4 - 97.7	--- ---
Burbelo *et al*. (2009): LIP or QLIPS	100[a]	100[a]	
Lipner *et al* . (2006): A rapid format anti -Ov-16 antibody test of whole blood or	81.1	99	Field-use friendly
Serum and plasma test	85.3	99	-do-
Boatin *et al*. (1998) Skin microscopy	19-50[a]	100[b]	---
DEC patch skin test	36-83[a]	98[a]	---
PCR ELISA	50-88	96[a]	---

Note: AGE; agarose gel electrophoresis, DEC; Diethyl carbamazine, PCHA; paper chromatographic hybridization assay. a: Interpreted from text. b: Stated as benchmark in text.

Nucleic acid-based tests: An alternative to skin mf detection definitive diagnostic test is the DNA molecular- based test, which is more sensitive and specific than the presence or and absence skin microfilariae (Boatin *et al*., 1998; Vincent *et al*., 2000). Ideally, the detection of any stage of the parasite or parasite products will unequivocally indicate current infection status. Other diagnostic approach is based on molecular biology techniques, which depends on detection of DNA or RNA fragment that are capable of distinguishing the parasite from other organisms. Due to minuteness of the nucleic acid (NA) molecule, the parasite-specific DNA and RNA oligo-nucleotide probes are amplified using polymerase chain reaction (PCR) to enhance the level of detection (Zimmerman *et al*., 1994; Rodriguez-Perez *et al*., 2004, Mekonnen *et al*., 2017). The nucleic acid amplification test (NAAT) of *onchocercal* complementary DNA or RNA primer sequences (O-150 base pairs) using PCR technique is dependent on skin snipping or scrapings, requires specialized skill, equipment and is costly. The test has proved to be more sensitive and specific (100%) than skin snips for mf (Zimmerman *et al*., 1994; Rodriguez-Perez *et al*., 2004). Comparatively a novel O-5S qPCR assay targeting the *O. volvulus* O-5S rRNA gene, had 100% specificity and proved more sensitive than O-150 qPCR assay (66.5% vs 39% positivity rate) (Mekonnen *et al*., 2017). Thus far, the various oligonucleotides that are specific for detection of *O. volvulus* have been produced and the list is many.

Nuclear genes may provide an alternative or companion to mitochondrial barcode sequences (Floyd *et al*., 2002). The most commonly used

barcode regions of nuclear DNA e.g. small subunit (ssu) and large subunit (lsu) rDNA—belong to multigene families, and although these are thought to exhibit concerted evolution, there are many cases where intragenomic variation has been detected, especially in the internal transcribed spacer regions e.g. ITS 1 and ITS 2 (Harris and Crandall, 2000; Chu *et al*., 2001). The forward primer has been used in a single PCR with a different reverse primer to produce 513 base pairs by (Nuchprayoon *et al*., 2005) while the reverse primer has been used in a second reaction of nested PCR to produce 344 bp amplification product by (Ta Tang *et al.,* 2010). The ribosomal genes are a family of tandem repeated units that despite concerted evolution do not necessarily show complete homogenisation.

One serious setback associated with nucleic acid (NA)-based molecular diagnostic methods was overcome by replacing the radioactive isotope detection of PCR products with enzyme-based reagents which has improved its field use and was envisaged to make it more adaptable for field application. DNA/RNA test using ELISA detection techniques required specialized skill, expensive equipment and costly reagents. Both methods do not fulfill the criteria of SMART. Detection of biotinylated PCR products by DNA probes was performed by ELISA to quantify the PCR product or by DNA detection test strips as a rapid field technique. Although the ELISA is theoretically more sensitive than the test strips for the detection of PCR products, examination of field samples revealed that the test strip method had a higher operational sensitivity and was more convenient to perform (Pischke *et al*., 2002). The DNA detection test strips were reported to be a rapid and low-technology tool for identification of PCR products in laboratories of countries endemic for onchocerciasis.

The complexity of these tests according to Alhassan *et al.* (2014) is the technical barrier which has been surmounted with the development of the loop-mediated isothermal amplification (LAMP). This technique amplifies DNA with high specificity, sensitivity and rapidity under isothermal conditions (Notomi *et al*., 2000). The LAMP reaction includes two sets of primers that hybridize to six sites on the target DNA, and a third set of primers (loop primers) to accelerate the reaction (Nagamine *et al*., 2001). The mixture of stem-loops containing alternately inverted repeats of the target sequence and cauliflower-like structures that are generated result in exponential amplification of the target sequence (>10 µg, >50 x PCR yield). Using three primer sets recognizing eight sites in the target DNA engenders the specificity to discriminate between genomic DNA at both genus and species-specific levels (Notomi *et al*., 2000; Nagamine *et al*., 2001; Nagamine *et al*., 2002, Alhasan *et al.*, 2014). LAMP employs Bst DNA polymerase, which provides both strand displacement and target amplification at a single temperature in a simple heat block or water bath at 60–65ºC as shown on Figure 4.

Enzymes and metabolites as diagnostic biomarkers: In similar vein, like antigens, immune responses to *Onchocerca* excretory or secretory enzymes have been measured to determine their reliability as diagnostic reagents. Among the enzymes so far tested includes alkaline phosphatase (E.C. 3.1.3.1) with a molecular weight of 90 kDa when in crude extract and dimerises to about 180 kDa upon purification. The enzyme was found to be secreted by both *O. ochengi* and *O. volvulus* worms. Sodium dodecyl sulphate at 2% (w/v) did not inhibit the enzyme activity, but apparently stabilized it during freezing. Inorganic phosphate inhibited the enzyme competitively with an apparent inhibition constant (K_i) of 3.33 ± 0.04 mM, whereas l-phenylalanine inhibited it in a mixed way with a K_i of 3.18 ± 0.03 mM. The apparently unique enzyme which is likely to serve in the nutrition of the parasite could be further characterized as a microfilaricide target or diagnostic marker in onchocerciasis (Cho-Ngwa *et al*., 2007). Another enzyme of interest is the *O. volvulus* Glutathione S-transferase 1a and 1b (*Ov*GST1a and -1b). These enzymes are unique GSTs in that they are glycoproteins possessing signal peptides that are cleaved off in the process of producing the mature protein. The mature protein starts with a 25-amino-acid extension not present in other GSTs. The ultrastructural localization of the secretory*Ov*GST1a and -1b in parts of the cuticle and in the outer lamellae of the hypodermis is consistent with the fact that they are secreted proteins (Liebau *et al*., 1994).

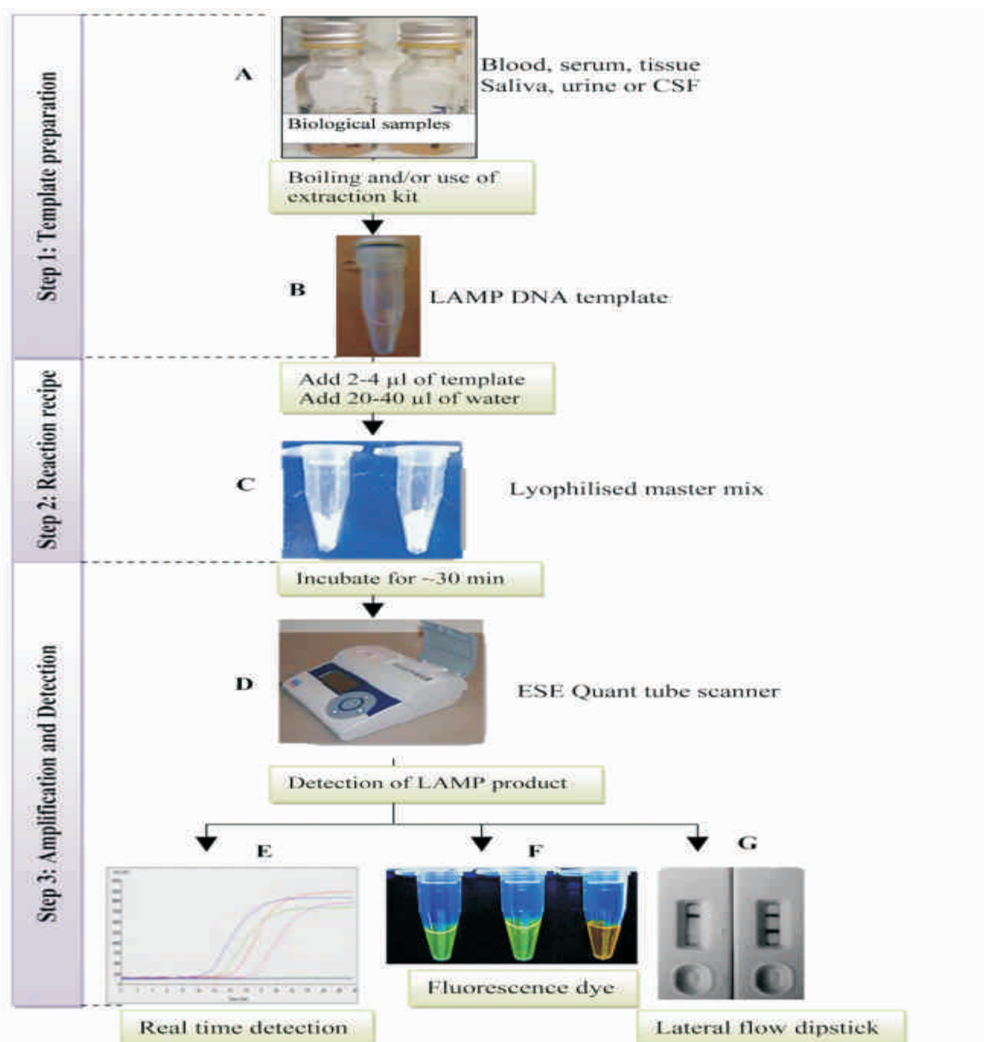

Figure 4: A proposed three-step LAMP method for diagnosing neglected tropical diseases. Step 1 includes processing of varied specimen (A) through boiling or use of kits to yield a stable and concentrated DNA template (B). In step 2, the lyophilised master mix (C) is reconstituted by the addition of water and the ideal amount of DNA template. In step 3, the amplification and the detection format are combined into a single step to avoid opening the tube (D), hence the results can be acquired in real time (E) through incubation of the reaction with a reporting dye (F) and through the use of a novel LAMP LFD format (Njiru, 2012).

The potential significance of *Ov*GST1a and 1b is serving as immuno-prophylactic targets and the immunological relevance of the N-glycans. This higher IgG reaction may be due to similar GST antigen epitopes, which might resemble the N-terminal portion of *Ov*GST1a. There is indication that the extracellular glutathione-*S*-transferase gene, *Ov-gst-1*, of *Onchocerca volvulus* has acquired a signal-peptide sequence (Sommer *et al*., 2001). This may be due to a hidden or former infection with *O. volvulus*. The serum pools from patients infected with the trematode *S. mansoni* did not react with the N-terminal extension peptides. Alhassan *et al.* (2014) reported on the identification of a new DNA biomarker, encoding *O. volvulus* glutathione S-transferase 1a (OvGST1a), and the development of a simple, single-step, LAMP assay that easily distinguishes between *O. volvulus* and *O. ochengi* DNA represents a significant technical advance.

Metabolomics, or the measurement of all the metabolites present in an organism, and metabolite profiling, in which a smaller subset of metabolites are measured, have become

established as useful tools in the "real-time" measurement of organismal metabolism (Vinayavekhin *et al.,* 2010). A metabolomic approach was applied to the discovery of biomarkers and to create a diagnostic test for identifying and classifying onchocerciasis infection using multivariate statistics and machine learning algorithms. The potential of metabolomic analysis has been demonstrated for uncovering biomarkers for specific determination of not only onchocerciasis infection but holds promise for the diagnosis of other parasitic diseases (Ritchie, 2006; Baek *et al*., 2009). One important biomarker is the *O. volvulus*-neurotransmitter tyramine (Globisch *et al*., 2013). Attempt to develop assays to detect potentially viable adult worms such as specific metabolites produced by female worms (Denery *et al*., 2010) include the test for parasite microRNA in the blood (Tritten *et al*., 2014, Quintana *et al*., 2015). The microRNA may not be present in sufficient concentration to act as a biomarker for infection (Latagie *et al*., 2017). Although this appeared to be sensitive and specific, but like the molecular test, it is time consuming, highly capital intensive and requires specialized skills. Therefore, it will be very difficult to domesticate enzyme and metabolite profiling procedures for field routine diagnostic application in DEC.

Importance of antibody based serodiagnosis of onchocerciasis: One of the major reasons for the immunology of any disease is to identify those molecules (antigens and antibodies) useful for serodiagnosis (Parkhouse and Harrison, 1989). Cross reactivity with other parasites was a major limitation that was overcome by using low molecular weight antigens (Weiss and Karam, 1989). Secondly, it was further enhanced by measuring IgE and IgG4 antibody (Weiss *et al*., 1982; Lal and Ottesen, 1988; Weil *et al*., 2000). Most of the cross reactants are high molecular weight carbohydrate moieties or glycoprotein containing ubiquitous immunodominant phosphorylcholine (PC). Moreover, IgG4 antibody does not react with PC molecule thereby conferring higher specificity to *O. volvulus* (Nde *et al*., 2005). Others have used various fractions of *O. volvulus* native antigens (Osue *et al*., 2008). Potential application of cocktail recombinant antigens in antibody detection ELISA has been emphasized by (Bradley *et al*., 1993 and Bradley *et al*., 1998). A four hybrid recombinant antigens resulted in increased reliability of assays compared to the individual antigens used (Andrews *et al*., 2008). Interesting, the hybrid antigens belong to the relatively low molecular weights band.

Screening and Confirmatory Tests for Monitoring Onchocerciasis Control: Antibody Card Test (Weil *et al*., 2000), the four-Ov-antigen standard or 15-minute format quick luciferase immunoprecipitation system (QLIPS) that has been found to show sensitivity and specificity of 100% (Burbelo *et al.,* 2005; Burbelo *et al.,* 2008; Ramanathan *et al*., 2008; Burbelo *et al*., 2009) cannot be overemphasized. Similarly, comparing it with the recent commercially available lateral flow test (Golden *et al*., 2013), a novel, inexpensive, and simple approach to actuating the detachment of the blood separation membrane from the nitrocellulose test with no impact on the performance characteristics of the test. The least DNA concentration above minimal detectable level as PCR can amplify down to 0.003 and 0.005 ng/μl, which corresponds to less than a microfilaria worm (Leroy *et al*., 2003). Secondly, the reliability of PCR to detect only few cases of infections (n=4) out of 64 samples after long-term treatment intervention (Osue *et al*., 2017) is in agreement with previous reports (Fink *et al*., 2011; Gopal *et al*., 2011 and Higazi *et al*., 2011). The sensitivity of PCR to detect the presence of microfilaria in skin snip sample has been found to be very high (Toe´ *et al.,* 1998). Where there is low infection during control and pre-infection period, PCR appeared to be more sensitive than reliance on presence of mf in skin snip, which are very scanty in the skin (Zimmerman *et al*., 1994; Lindblade *et al*., 2007; Cruz-Ortis *et al*., 2012).

Problems Inherent in Molecular Based Tests for Assessing Onchocerciasis Control: It is therefore expedient to caution the sole dependence on parasitological test to declare sample population free of microfilaria as previously observed (Tekle *et al.,* 2012; Osue *et al.,* 2013). Similarly, Convit *et al.* (2013) used PCR amplification and antibody reaction with *O. volvulus* antigens to support assessment of

onchocerciasis control in female insect head and school children in endemic areas of Northern Venezuela. The primer sequences used in this study, 18S ITS-1 gene gave both single and double band in DNA templates of two positive samples. The controvertible outcome of ITS1-PCR double amplicons is subject to further research. Implication of the forward primer also found to be located in a fungi family *Cortinariaceae* 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence (Liimatainen *et al*., 2014) remained unanswered. The fungus has been reported to belong to Agaricales, GenBank accession No. gbKF732628 which is regarded as the largest family of fungi.

The rRNA ITS1 gene region analysis provides a multi-species-specific diagnosis by a single PCR (Salim *et al*., 2011). Furthermore, previous reports (Njirun *et al.,* 2005; Cox *et al,* 2005) documented specific PCR product length corresponding to each *Trypanosoma species*, which was the basis for differentiation. In an experiment to detect coagulase (coa) gene, the PCR products showed double bands, three bands and single PCR band (Tiwari *et al*., 2008). Secondly, available evidence from the GenBank database, the ITS1 genes are in two or more loci. Therefore, it is very likely that some DNA amplifications could give rise to double band amplicons either due to presence of different loci. The observed double bands in gel electrophoresis, which re-occurred in a repeated PCR assay, appeared to be a unique characteristic that have never been reported for *O. volvulus* or other filarial species. In addition, nonspecific amplification may occur at other sites with similar sequences (Vierstracte, 1999) or reduced amplification may occur at primer-template mismatched sites. Another study by Higazi *et al*. (2001) in Sudan revealed that *O. volvulus* endemic to the northern focus at Abu Hamed were significantly different from all other *O. volvulus* populations examined to date. There is documented evidence for ITS1 PCR amplification, which resulted in a double-band pattern in all *Leishmania tropica* cases, while a sharp single band was observed for *L. infantum* and *L. major* isolates (Schonian *et al*., 2001; Ghatee *et al*., 2013). The double-band pattern was attributed to a 100 bp fragment difference within ITS-rDNA alleles. Thirdly, the BLASTN analysis from the sequences derived from both the forward and reverse sequences generated did not show any significant alignment with mega blast for *O. volvulus* nucleotides in the GenBank database. But there was somewhat similar alignment with *O. volvulus* genomic DNA from Cameroon isolates. More importantly, not much reports on primers designed available are from Nigeria parasites. Therefore, whether this is due to new sequences that have not been reported is subject to further investigation.

The outcome of both PCR and sequencing that confirmed the amplicons were those of *O. volvulus* proved that ITS1 primers used have greater positive predictive value than skin snip microscopy. A similar finding (Fink *et al,*2011) using real time PCR with 2 out of 218 samples positive by microscopy and PCR and 12 samples were positive by PCR only. PCR based assays targeting specific genomic sequences are theoretically 100% specific. In practical terms, PCR assays are subject to false positive results that are caused by contamination with amplicons. This obstacle can be overcome by employing a confirmatory assay that targets an independent portion of the target organism's genome. However, in individuals with low mf densities, whose skin samples may be devoid of whole or partial mf, the PCR will be negative because free parasite DNA is not stable in the tissue (Fischer *et al*., 1996). Therefore, the PCR on tissue samples is a sensitive, direct method to prove the presence of intact, disintegrating mf or remnants of them. This brings us to the question whether a gene copied during PCR was the right sizefi According to Vierstracte (1999), some problems that make every PCR not to be successful are probably due to the poor quality of the DNA, that one of the primers doesn't fit, or that there is too much starting template. Secondly, is the product the right size as the band of 500 base pair, and the expected gene for *O. volvulus* should be 344 bp molecular weight (Morales-Hojas *et al.*, 2010). It could be possible one of the primers probably fits on a part of the

gene is either closer or farther to the other primer. It is also possible that both primers fit on a totally different gene. Thirdly, where more than one band is formed, it is possible that the primers fit on the desired locations, and also on other locations. In that case, you can have different bands in one lane on a gel. Moreover, the *Taq* polymerase used has no proofreading function (3'-5' exonuclease activity), therefore is prone to generate errors during DNA synthesis (Suchman, 2013). Other thermo stable archeal DNA polymerases such as *Pfu* which has 3'-5' proofreading function can be used to overcome such problem.

Field Application of Screening and Confirmatory Tests for Surveillance at Post-control: A combination of screening tool, standard skin snip microscopy of emerged microfilaria and application of LAMP assay for the amplification of specific target DNA detectable using turbidity or by a hydroxyl naphthol blue colour change described previously (Goto *et al.,* 2009; Alhassan *et al.,* 2014) may provide a better epidemiological situational assessment for the determination of control and certification of eradication status of ivermectin treated communities. The results indicated that the assay is sensitive and rapid, capable of detecting DNA equivalent to less than one microfilaria within 60 minutes. A greater positive predictive value of the screening test over the two diagnostic tests could be attributed to false positive rate. Predictive values are not stable characteristics of a diagnostic test (Elavunkal and Sinert, 2007) and are dependent on disease prevalence. The same diagnostic test will have varying predictive values in different populations. Both conventional and real-time PCR-based assays (Figure 4) are significantly more sensitive than current methods for diagnosing *Onchocerca volvulus* infection thereby overcoming many of the difficulties in identifying active onchocerciasis (Nutman *et al*., 1996). Fink *et al.* (2011) averred that since chemotherapy is widely used to treat onchocerciasis, PCR would be of immense importance to assessing responses to treatment, predicting recrudescence and detecting filarial infections in mobile populations. Cases of positive reaction among the infected and non-infected malaria control sera from participants are not indicative of cross reaction.

Field Friendly Screening Tools and Laboratory Based Validation Techniques: Among the possible tools available for field use are the recently patented by SD-BIOLINE (Alere Standard Diagnostics, Inc., Group of Companies, South Korea). SD-Onchocerciasis IgG4 is a rapid test for the detection of IgG4 antibodies against Ov16 antigen (SD-Bioline, 2015). This is a rapid qualitative test for detecting IgG4 antibodies against *O. volvulus* antigens in human finger-prick blood, serum and plasma. Result can be obtained within 20 minutes. The shortcoming of this screening test is the inability to differentiate active from past exposure to infection. In a study, sentinel cohort in communities, 12% (15/126) were serologically positive for antibodies recognizing a cocktail of *O. volvulus* recombinant antigens in El Ámbar, while 7% (5/68) were serologically positive in Altagracia. The incidence in children five years old and younger (*n* = 27) within this sentinel cohort was 13% (2/15) in ElÁmbar, and 8% (1/12) in Altagracia. Table 1 shows the cases of antibodies in patients after long period of treatment.

A PCR with *Onchocerca* specific primers is used to reproducibly detect one heavily infected blackfly in a pool of 80 flies, or to detect one blackfly inoculated with one microfilaria in a pool of 20 flies (Oskam *et al*., 1996). With the pool screening method described, large numbers of blackflies can be rapidly screened for the presence of *0. volvulus* infected flies. This procedure has been validated in field studies in African and South America by Guevara *et al*. (2003), Poolman and Galvani, (2006) and Marchon-Silva *et al*. (2007) and found very reliable in testing large number of blackflies for infection rate compared to the classical microscopic dissection method to detect active cases in a fly population. This approach is beset with the problem of not actually knowing if more than one fly per pool of screened flies were infected.

When to Stop MDA and How to Confirm Post-Treatment Recrudescence: Two crucial issues about the MDA with ivermectin revolve principally around how to determine when to stop MDA.

Secondly, also the need for continued surveillance to monitor and evaluate if there is possible emergence of treatment failure as reported in Ghana by Awadzi *et al*. 2004 and Osei-Atweneboana, *et al*., (2011). Any of the two situations will dependably rely on applying combined screening and diagnostic tools that meet the criteria with the acronym ASSURED; Affordable, Sensitive, Specific, User-friendly (simple to perform in a few steps with minimal training), Robust and rapid (results available in 30 min), Equipment free, and Deliverable to the end user (Mabeh *et al*. 2004, Njiru, 2012). Overall, due consideration should be given to the WHO guidelines to determine when to stop MDA based on the results of entomological (O-150 PCR Pool-screen) and serological (Ov-16) testing as summarized in a decision tree (Figure 5). It is strongly recommended to undertake these tests 12 months after the last round of MDA and during peak period of parasite transmission (WHO, 2016). The DECs should follow guidelines in the decision tree for stopping MDA as elimination programmes transition from phase 1 (treatment) to phase 2 (post-treatment surveillance) using both PCR in black fiies and serology in children aged under10 years. After a PTS period of 3–5 years the interruption of transmission is confirmed from entomological (O-150 PCR Pool-screen) test and or serological (Ov-16) testing as shown in the algorithm of PCR testing depicted in a flow chart (Fig. 6). Elimination status can only be granted to a country by the WHO Director-General when all detected foci under long-term, continuous ivermectin treatment are certified to be free of transmission, and all areas of potential transmission of *O. volvulus* can be sufficiently excluded not to occur any longer in that country after the report of the international verification team.

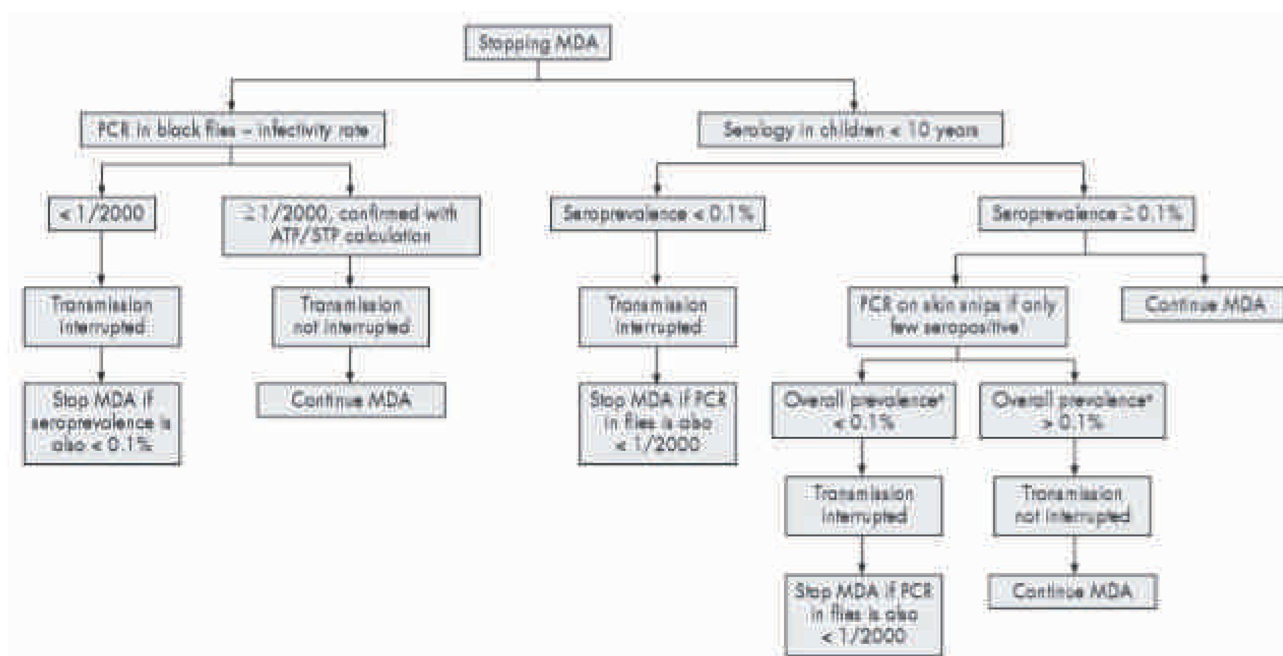

Figure 5: ATP, annual transmission potential; MDA, mass drug administration; PCR, polymerase chain reaction; PTS, post-treatment surveillance; STP, seasonal transmission potential 1 Few is defined here as below 10. Overall prevalence: the number of seropositive children minus the number of seropositive children who tested negative at PCR on skin snips, divided by the number of children who were tested for serology. Source: WHO, 2016.

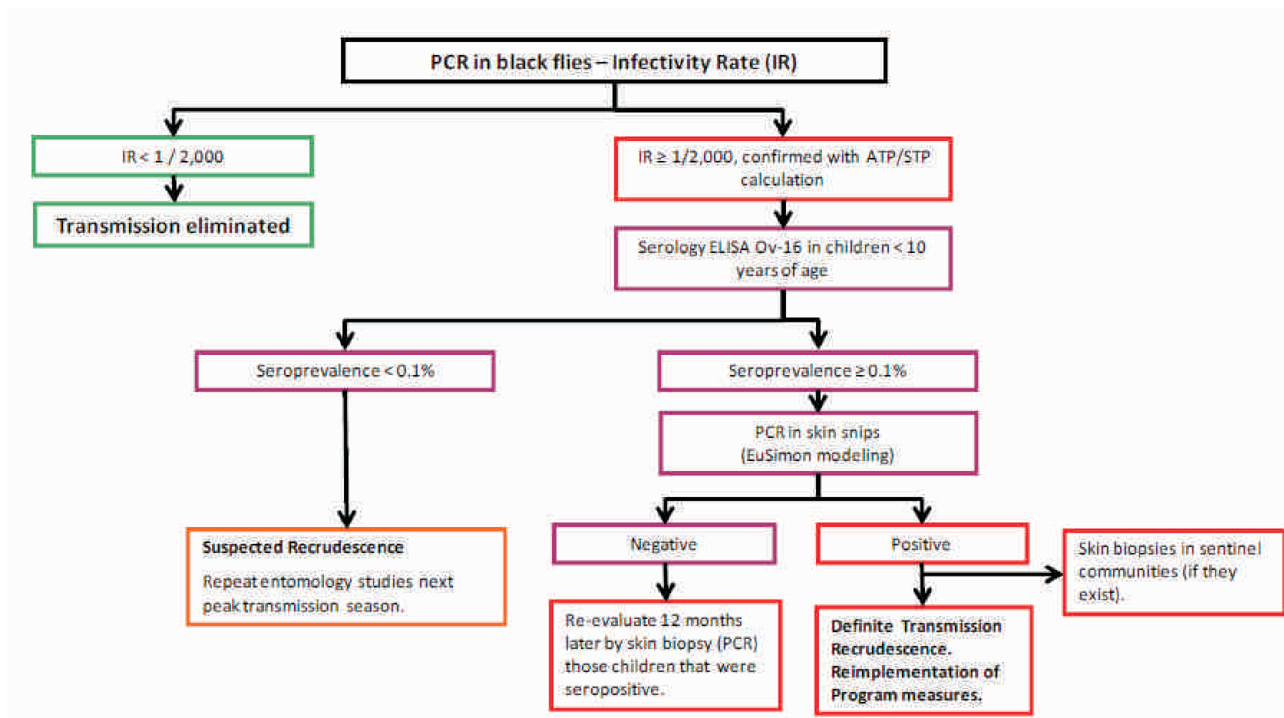

Figure 6: Post Treatment Surveillance Decision Tree for the Detection, Confirmation, and Response to a Potential Recrudescence Event. ATP/STP: annual or seasonal transmission potential. Source: PAHO/WHO, (2011). Source: Colorado School of Public Health, power point presentation citing Udall (2007).

Conclusion:
It is a matter of considerable importance and expediency to combine any of the screening (serological SD-BIOLINE and DEC-PST) and molecular diagnostic PCR tests based on different Bst primer sequences in LAMP in determining the disease elimination status of the DECs. Reliability and accuracy of all these tests had decreased drastically with the increased level of treatment dosage and demographic and geospatial coverage of human population over a long time period. But in the case of assessing the blackfly infection rate, the highly recommended pool-screen test remained the best option despite the inability to detect the actual number of flies that are infected within a positive pooled test sample. Any positive case in pool-screen test is an indication of continued transmission but its absence does not equivocally rule out its presence. Hence, the need to continually carry out the mandatory surveillance for the stipulated period of 3-5 years post-treatment intervention and at the peak of transmission in large population of black flies and in children less than 10 years of age based on the WHO guidelines should strictly adhered to. Human capacity building and institutional capability strengthening will be required to domesticate O-150 PCR and LAMP to empower DECs carry out the meticulous and arduous tasks of performing the highly accurate, highly thorough-put and active surveillance across the disease endemic communities.

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