www.ajbrui.org

Afr. J. Biomed. Res. Vol. 21 (January, 2018); 23- 28

Research article

Onchocercal DNA Amplification Using Beta Actin Gene Primers Compared with First Internal Transcribed Spacer Sequences for Monitoring Onchocerciasis Eradication Strategy

***Osue, H.O[1], Inabo, H.I[2], Yakubu, S.E[2], Audu, P.A[3], Mamman, M[1]**

[1]*Research Planning, Monitoring, Extension, Statistics, and Socio-economics Department, Nigerian Institute for Trypanosomiasis (and Onchocerciasis) Research, P. M. B. 2077, No. 1 Surame Road, Unguwar Rimi GRA, Kaduna, Kaduna State, Nigeria.*

[2]*Microbiology Department, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna State, Nigeria 3*

[3]*Department of Biological Science, Faculty of Science, Federal University of Lokoja, Lokoja, Kogi State, Nigeria*

ABSTRACT

Ongoing treatment control strategy against onchocerciasis or river blindness will need efficient diagnostic method to evaluate the mass drug administration with ivermectin (Mectizan®). Sole reliance on classical parasitological method of skin snip microscopy for detecting microfilaria has proved less sensitive during post-control period. Detecting any parts of the parasite stages such as antigens, enzymes and nucleic acids (DNA and RNA) is a definitive diagnosis and highly sensitive. This study was to evaluate the diagnostic reliability of the beta actin gene primer pair to confirm its suitability for validating presence or absence of skin microfilaria at post-treatment. DNA extracted from skin snip samples (n=15) from an onchocerciasis mesoendemic area, three from non-endemic, two adult worm fragments and blank wells with only master mix (n=7) were subjected to endpoint polymerase chain reaction (PCR) analysis. Four of the samples had shown reactivity with first internal transcribed spacer (ITS1) primer pair. The amplicons were sequenced and subjected to basic local alignment search tool (BLAST). Out of the 12 amplicons in agarose gel, there were 6 sharp and 6 faint bands of 100bp molecular weight as documented. The sharp bands included 3 ITS1 and one field positive samples, and 2 positive controls. The BLAST analysis showed moderate homology with beta actin with accession number M84916 available in the public GenBank database, and with the positive control sequences. This study has shown that DNA amplification with beta actin gene may be very specific and more sensitive compared with the ITS gene primer sequences.

Keywords: *Beta actin, DNA amplification, Onchocerca volvulus, polymerase chain reaction; sequence alignment, skin microfilaria*

*Author for correspondence: *E-mail*: osueho@yahoo.com; *Tel: +234(0)8076779890*

Received: September, 2017; Accepted: December, 2017

Abstracted by:
Bioline International, African Journals online (AJOL), Index Copernicus, African Index Medicus (WHO), Excerpta medica (EMBASE), CAB Abstracts, SCOPUS, Global Health Abstracts, Asian Science Index, Index Veterinarius

INTRODUCTION

Definitive diagnosis of onchocerciasis is based on detection of skin or ocular microfilaria and presence of adult worm from extirpated nodule or onchocercomata. Presence of one or more clinical signs such as leopard skin, elephantiasis of the extremities, hanging groin, and eye visual impairment and blindness are strongly indicative of suspected cases. Outline of the WHO (2016) guideline to determine the eradication and elimination of onchocerciasis are based on criteria that spelt out four phases to determine attainment of the goal are: (i) transmission suppression; (ii) interruption of transmission; (iii) precertification that required post-treatment evaluation, and (iv) declaration of elimination may not rely solely on microscopic detection of mf in parous flies or human host (Cruz-Ortiz et al., 2012). Rodriguez-Perez et al., (2004) have placed high premium on polymerase chain reaction (PCR) assay for monitoring mass drug treatment with ivermectin. A combination of screening tool, standard skin snip microscopy of emerged microfilaria and application of LAMP assay for the amplification of specific target DNA that can be detected using turbidity or by a hydroxyl naphthol blue colour change described by Goto et al. (2009) and Alhassan et al. (2014) will provide a better epidemiological situational assessment for the

determination of control/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. In addition, the LAMP has been adjudged by Poole et al. (2012) to be relatively cheaper than the PCR and RT-PCR.

Very sensitive diagnostic tool is especially necessary in order to confirm that transmission has been interrupted and determine the correctness of data on the epidemiological situation of onchocerciasis should not be basedmainly on microfilaria (MF) skin snip assessments (Vlaminck et al., 2015; Moya et al., 2016). An alternative to skin mf detection is the DNA molecular based test, which is more sensitive and specific than the skin microfilariae detection (Boatin et al., 1998b; Vincent et al., 2000). Ideally, the detection of any stage of the parasite or parasite products will unequivocally indicate current infection status. The nucleic acid amplification test (NAAT) of Onchocercal complementary DNA or RNA primer sequences 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) are capable of distinguishing the parasite from other organisms. Various oligonucleotides that are specific for detection of O. volvulus have been produced. 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.

MATERIALS AND METHODS

Sample collection:
Extracted DNA from skin snip sample (n=15) obtained from residents (n=64) in the study areas. The DNA from skin snips of persons living in onchocerciasis non-endemic areas served as negative control (n=3) were tested in PCR assay performed in a 50-µl reaction buffer and mastermix as described by Osue *et al*. (2017). The primers used satisfied the criteria given for selecting sequence primers by the kit manufacturer, QIAGEN®, Germany. The PCR was performed at the International Institute for Tropical Agriculture (IITA) Molecular Biology Laboratory, Ibadan. Amplification was performed in 30 cycles at 94° C to denature, 55°C annealing and 72 °C extension of 30 s each. In PCR assays included wells that contained adult worm *O. volvulus* DNA fragment, mastermix with water instead of sample DNA and three skin microfilaria negative, which served as positive, internal assay (blank wells) and negative controls, respectively.

Primer sequences:
The PCR products were amplified using filarial-specific DNA of *Onchocerca volvulus* actin-2B (Embank accession no. M84916) described by Gilbert *et al*. (2005) specific primers; forward- GTGCTACGTTGCTTTGGACT and reverse-GTAATCACTTGGCCATCAGG were used to amplify DNA samples (n=15), which included 4 PCR ITS1 positive.

Gel electrophoresis:
Amplified DNA products were subjected to agars gel electrophoresis at 2% w/v gel over TBE buffer and 10µl Sober Green as described by Osue *et al*. (2017). Briefly, a 15 all sample was mixed with 2 all of loading dye were carefully loaded into wells created by the combs using a 20 all pipette tip. Electrophoresis was run at 60-100V till migration of dye reached three-quarter of gel. Gel was observed under a UV Trans-illuminator and the molecular weight band documented in a Signee Dig genius Gel Documentation System with camera linked to a computer.

PCR product extraction and sequencing:
Bands of DNA in gel were cut, melted in Strata gene Gradient PCR and amplified products were extracted using QIAGEN® PCR Purification Kit essentially as described by the manufacturer. Amplification of PCR products were carried out using the same forward and reverse primers. The BigDye termination sequencing with chain terminator ddNTPs was used in a single reaction. Each of the four dideoxynucleotide chain terminator was labelled with a fluorescent dye, which emit light at different Wavelength as recommended in the manufacturer's instruction manual. Sequencing of the PCR products were carried out with SDS-PAGE Beckman Coulter CEQ 2000XL DNA Analysis System at the DNA Laboratory, Kaduna.

Analysis of primer sequence blast hits:
The National Centre for Bioinformatics (NCBI) search engine was used to perform BLASTn. Sequences were aligned using CLUSTALW (Thompson *et al*., 1997) Multiple Sequence Alignment Program, Version 1.7(EMBL, Heidelberg) for the primers sequences of *O. volvulus*. Also, analyses of data were generated to see the percentages and sequence length or number of nucleotide similarities scored was subjected to statistical analysis. Frequency of homology or similarity with sequences from sister (animal infective) *Onchocerca* species, other filarial and non-organisms were compared.

RESULTS

PCR amplicons: Samples of skin snip DNA extract obtained from a mesoedemic onchocerciasis area undergoing ivermectin treatment for 18 years that had been previously subjected to ITS1 PCR analysis were tested with beta actin gene primer sequence DNA amplification. The DNA extracts tested comprised field samples (n=15), three negative and two positive control. Amplification was done twice. The first PCR assay had seven wells that contained master mix without templates served as assay control. Twelve samples (n=12) with amplification bands in agarose gel electrophoresis (Plate 1A and B) included four that had shown reactivity with ITS1 primer pair sequences and the two positive control adult worm extract. The 12 amplicons showed 7 sharp and 5 faint bands. The sharp bands included the 3 ITS1 PCR positive samples and 2 positive controls (P1 and P2) and 2 other field samples. None of the seven assay control wells were amplified.

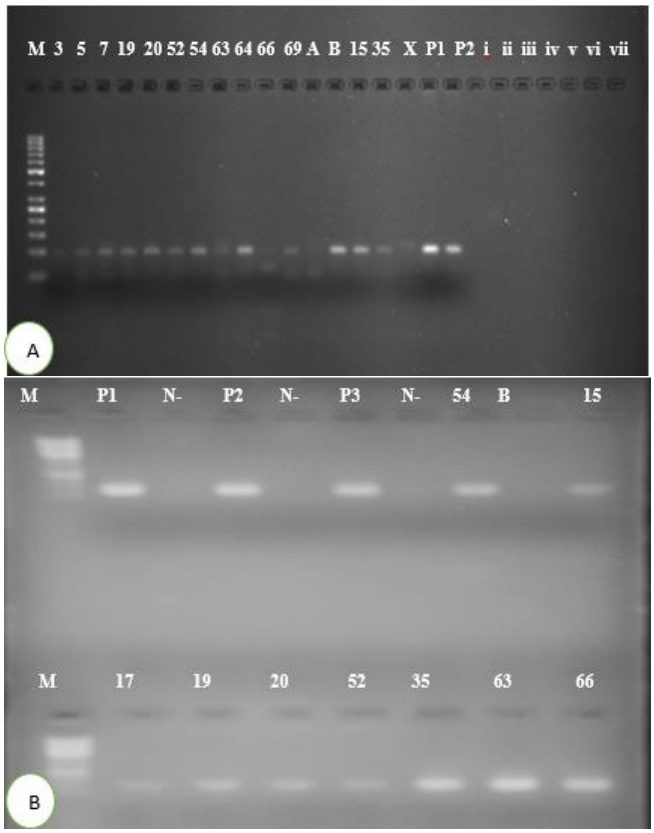

Plate I:
Gel electrophoregram of PCR amplicons of genomic DNA with beta actin gene primers. (A) The DNA templates (n=18) included field samples (n=13), which is inclusive of the ITS1 PCR positive samples (n=4), positive control (n=2) and negative control (n=3). The blank wells (i - vii) contained master mix without template. The molecular (M) weight ladder is 1kilobase in multiples of 100bp. (B) Repeated DNA amplification of the samples. The sharp bands included the 2 positive controls (P1 and P2) and the 3 ITS1 (35, 63 and 66) PCR positive samples

Molecular weight: The molecular weight of the amplicons of about 100bp was the same with those documented in literature for beta actin gene. The sensitivity of the actin 2B gene primer sequence on Table 1 were 60%. The six DNA extracts that were confirmed to be ITS1 PCR positive consisted of four field samples and two fragments of female adult worms were all amplified (100%).

Sequences alignment: The DNA sequences subjected to basic local alignment search tool (BLAST) analysis showed 60% alignment with GenBank Accession Number ID: gb|M84916 located at 1033-1052 and 1131-1120 (99bp) or 1675-1694 and 1773-1754 as shown on Figure 1. The sample sequence identity with that of the control had 68% homology identity (Figure 2). The consensus sequences showed the point gaps and area of nucleotide similarity.

Table 1:
PCR Sensitivity and specificity analysis of actin 2B gene Primer sequences

S/No.	Test	Sample size (n)	PCR positive (%)
1	Field samples*	15	10 (60.0)
2	ITS1 PCR Positive*	4	4 (100)
3	Positive control	2	2 (100)
4	Negative control	3	0 (0)
5	Internal control	7	0 (0)

** The ITS1 samples (n=4) were among the field samples*

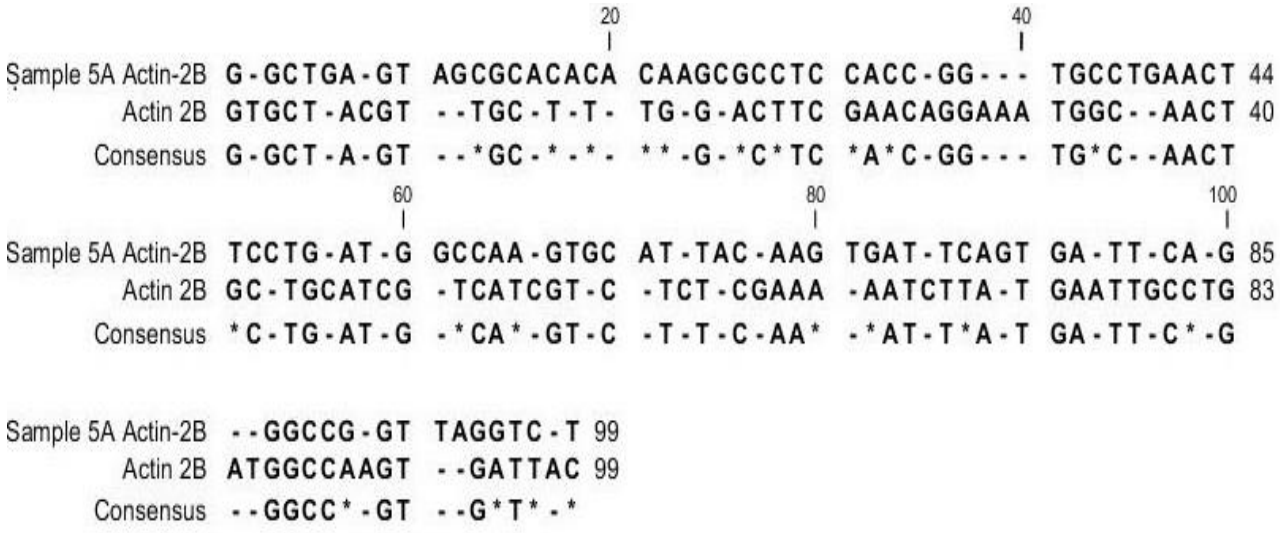

Fig. 1:
DNA sequence alignment of sample 15 and O. volvulus Beta Actin (Actin-2B) gene (A) and positive control 2 (B) using ClustalW. A: Consensus sequence with (-) and (*) signs representing gaps and points of conflict, respectively. There were about 60% alignments with GenBank Accession Number ID: gb|M84916 l.

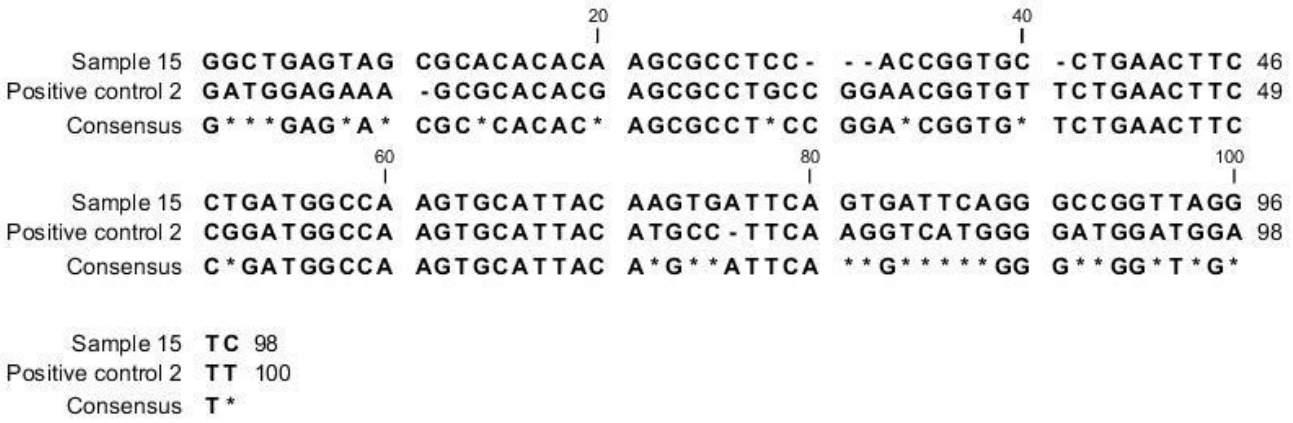

Fig. 2:
DNA sequence alignment of sample consensus sequence showed 68% homology between sample 15 and positive control using ClustalW. Consensus sequence with (-) and (*) signs representing gaps and points of conflict, respectively.

DISCUSSION

Both conventional and real-time PCR-based assays are significantly more sensitive than current methods for diagnosing *Onchocerca volvulus* infection, and it overcomes many difficulties in identifying active onchocerciasis (Nutman *et al*., 1996). Fink *et al.* (2011) averred that since chemotherapy is widely used to treat onchocerciasis, PCR is important for assessing responses to treatment, predicting recrudescence and detecting filarial infections in mobile populations. This study showed that DNA amplification with beta actin gene may be detected in samples of onchocerciasis after long–term ivermectin treatment. It seems to be very sensitive and equally specific when compared with the ITS gene primer sequences. Superior sensitivity of molecular diagnosis using primers of conserved ITS1 18S ribosomal DNA (Osue *et al*., 2017) and actin-2B genes for PCR amplification of template invalidated the zero prevalence of skin microfilaria as observed in the main study from where the samples were drawn (Osue *et al*., 2013). This study has practically demonstrated that the actin-2B gene specific primers seems to be more sensitive than the ITS1 sequences with the higher number of PCR positive recorded. On the hand, the observed difference cannot be attributed to cross-reaction with host actin-2Bgene presumed to differ in nucleotide sequence identity.

The sensitivity of PCR to detect the presence of microfilaria in skin snip sample based on amplification of DNA (Figure 1) was found to be very high is in agreement with Toe´ *et al.* (1998). The findings from this study has confirmed the probable presence of microfilaria in the population (n=592) reported to be skin snip microfilaria negative by Osue *et al*. (2013). It is therefore expedient to caution sole dependence on parasitological test to declare sample population free of microfilaria (Tekle *et al* 2012; Osue *et al* 2013; Lloyd *et al*. 2016). Importance of PCR during post-control and pre-infection period proved to be more sensitive than presence of mf in skin snip, which are very scanty (Zimmerman *et al*., 1994; Lindblade *et al*., 2007; Cruz-Ortiz *et al*., 2012). This corroborate the finding by Moya *et al*. (2016) where they recorded a seroprevalence with IgG4 serology of 7.9%. No children less than 10 years old were found to be positive for the test. Only one case was positive for *O. volvulus* after skin PCR test. Accor ding to Lloyd *et al*. (2015) improved methods are needed for those areas with lower infection prevalence, which might be close to elimination and cannot be identified based on rapid epidemiological mapping of onchocerciasis (REMO).

Individuals with low mf densities, whose skin snip are devoid of whole or partial mf PCR will invariably 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 the remnants. Both ITS1 and mitochondria associated primer sequences appeared to have surmounted the problem of cross-reactivity of genomic O-150 bp DNA sequences with other filarial worms (Meredith *et al*., 1991; Zimmerman *et al*., 1994). This disparity is due to the fact that the genomic DNA is fewer compared to mitochondria and ribosomal DNA that are more abundant (Robin and Wong, 1988; Ferri*et al*., 2009; Lefoulonet *al*., 2015). Therefore, this might explain why the beta actin is more sensitive than the ITS1 ribosomal conserved DNA primer sequences used in this study.

The sensitivity of actin-2B primers have been confirmed with the amplification of all the ITS1 PCR positive and two positive controls. There is no doubt that the non-amplification of the negative and blank well has confirmed the specificity and excluded the probability of contamination, as it was very unlikely in this circumstance. Secondly, and more importantly, on Fig.2, the level of similarity or identity of sample sequences with O. *volvulus* beta actin gene specific DNA sequences available in the GenBank database and the positive control sequence have clearly verified the integrity of the amplicons to be *O. volvulus* specific. Sequence analysis identified conserved sites in the 18S and 5.8S rDNA sequence that could be used as universal priming sites to generate ITS1-distinctive PCR products that were useful for distinguishing

filarial at the genus level. Outcome of this study has reinforced the need for the establishment of an algorithm for surveillance and monitoring onchocerciasis and sympatric filarial infection to determine when to stop treatment according to WHO (2016) guidelines. A combination of antibody detection, single PCR analysis with O-150bp sequences and the gold standard for confirmation deserved further research when building capacity to establish national reference laboratory to monitor and evaluate ongoing CDTI to determine when control and eradication or elimination statuses attained after long-term post-treatment. No doubt, PCR assay will be needed to confirm the rapid diagnostic test positive cases particularly in children living within endemic communities during impact assessment.

With the new molecular method of loop-mediated isothermal amplification (LAMP) has been adapted as an alternative field based molecular technique that amplifies DNA with high specificity, sensitivity and rapidity under isothermal conditions (Notomi *et al.*, 2000; Nagami *et al*., 2002). 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 (Nagami *et al*., 2001). Amplification of specific target DNA is detected using turbidity or hydroxyl naphthalol blue colour change (Goto *et al*., 2009; Alhassan *et al*., 2014). Recently, LAMP method was designed using the mitochondrial *O. volvulus* cox1 gene as a target (Lagatie *et al*., 2016).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. It might be correct to make fiducial statement that actin 2B primer sequence may be a high candidate to be considered for use in LAMP assay

Acknowledgements

The authors are grateful to the Management of NITR for sponsoring H. O. Osue and for funding this project, which was part of his PhD thesis. The services of DNA Laboratory, Kaduna and Biotechnology Laboratory, International Institute for Tropical Agriculture, Ibadan, Oyo State, Nigeria for the PCR assays

REFERENCES

Alhassan, A., Makepeace, B.L., LaCourse, E.J., Osei-Atweneboana, M.Y. and Carlow, C.K.S. (2014). A Simple Isothermal DNA Amplification Method to Screen Black Flies for *Onchocerca volvulus* Infection. *PLoS ONE* 9(10):e108927.doi: 10. 1371/ journal.pone. 0108927.

Albers, A., Esum, M. E., Tendongfor, N., Enyong, P., Klarmann, U., Wanji, S., Hoerauf, A. and Pfarr, K. (2012). Retarded *Onchocerca volvulus* L1 to L3 larval development in the *Simulium damnosum* vector after anti-wolbachial treatment of human host. Parasites and Vectors, 5: 12- doi: 10.1186/1756-3305-5-12

Alschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Research, 25: 3389-3402.

Ardelli, B.F. and Prichard, R.K. (2004). Identification of variant ABC-transporter genes among *Onchocerca volvulus* collected from ivermectin-treated and untreated patients in Ghana, West Africa. *Annals of Tropical Medical Parasitology*, 98:371–384.

Boatin, B.A., Toé, L., Alley, E.S., Nagelkerke, N.J., Borsboom, G. and Habbena, B.A.L. (2002). Detection of *Onchocerca volvulus* Infection in Low Prevalence Areas: a Comparison of Three Diagnostic Methods. *Parasitology*, 125:545-552.

Casinader Saverimuttu, J. K., Karunanayake, E. H., Chandrasekharan, N. V. and Jayasena, S. M. (2000). Molecular characterisation of the actin gene of the filarial parasite*Wuchereria bancrofti*. *International Journal of Parasitology*, 30: 119–124.

Chu, K.H., Li, C.P. and Ho, H.Y. (2001). The First Internal Transcribed Spacer (ITS-1) of Ribosomal DNA as a Molecular Marker for Phylogenetic and Population Analyses in *Crustacean*. *Mar Biotechnology*, 3:355–361.

Cruz-Ortiz, N., Gonzalez, R. J., Lindblade, K. A., Richards, F. R., Saurbrey, M., Zea-Flores, G., Dominguez, A., Oliva, O., Catu, E. and Rizzo, N. (2012). Elimination of *Onchocerca volvulus* Transmission in the Huehuetenango Focus of Guatemala. Hindawi publishing Cooperation *Journal of Parasitology Research*, Article ID 638429, 9 pages.

Eng, J. K. L. and Prichard, R. K. (2005). A comparison of genetic polymorphism in populations of *Onchocercavolvulus* from untreated- and ivermectin-treated patients. Molecular & Biochemical Parasitology 142 (2005) 193–202.

Fink, D.L., Fahle, G.A., Fischer, S., Fedorko, D.F and Nutman, T.B. (2011). Toward Molecular Parasitologic Diagnosis: Enhanced Diagnostic Sensitivity for Filarial Infections in Mobile Populations. *Journal of Clinical Microbiology*, 49(1):42-47.

Ferri, E., Barbuto, M., Bain, O., Galimberti, A., Uni, S., Guerrero, R., et al. (2009). Integrated taxonomy: traditional approach and DNA bar-coding for the identification of filarioid worms and related parasites (Nematoda). Front Zool. 6:1.

Fischer, P., Rubaale, T., Meredith, S.E.O. and Butter, D.W. (1996). Sensitivity of a PCR-Based Assay to Detect *Onchocerca volvulus* DNA in Skin Biopsies. *Parasitology Research*, 82:395–401.

Gilbert, J., Nfon, C. K., Makepeace, B. L., Njongmeta, L. M., Hastings, I. M., Pfarr, K.M., Renz, A., Tanyan, V. N. and Trees, A. A. (2005). Antibiotic chemotherapy of onchocerciasis: in a bovine model, killing of adult parasites required sustained depletion of endosymbiotic bacteria (*Wolbachia* species). *Journal of Infectious Diseases*, 192: 1483- 1493. Doi: 10.1086/46246.

Goto, M., Honda, E., Ogura, A., Nomoto, A. and Hanaki, K. (2009). Colorimetric Detection of Loop-Mediated Isothermal Amplification Reaction by Using Hydroxyl Naphthol Blue. *Biotechniques*, 46:167–172.

Lagatie, O., Merino, M., Debrah, L.B., Debrah, D.Y. and Stuyve, L.J. (2016). An isothermal DNA amplification method for detection of Onchocerca volvulus infection in skin biopsies. Parasites & Vectors. 9:624. DOI 10.1186/s13071-016-1913-7

Lefoulon, E., Bain, O., Bourret, J., Junker, K., Guerrero, R., Canizalesm I., *et al*. (2015). Shaking the tree: multi-locus sequence typing usurps current onchocercid (filarial nematode) phylogeny. PLoS Negl. Trop. Dis. 9(11):e0004233.

Lindblade, K.A, Arana, B., Zea-Flores, G., Rizzo, N., Porter, C.H., Dominguez-Vasquez, A., Cruz-Ortiz, N., Unnasch, T.R., Punkosdy, G.A., Richards, J., Sauerbrey, M., Castro, J., Catú, E., Oliva, O. and Richards, F.O. (2007). Eradication of *Onchocerca volvulus* Transmission in the Santa Rosa Focus of Guatemala. *American Journal Tropical Medicine and Hygiene*, **77**:334–341.

Lloyd, M.M., Gilbert, R., Taha, N.T., Weil, G.J., Meite, A., Kouakou, I.M.M., *et al*. (2015). Conventional parasitology and DNA-based diagnostic methods for onchocerciasis elimination programmes. Acta Trop. 25: 146:114–8. Doi: 10.1016/j.actatropica. 2015.03.019.

Meijerink, J., Mandigers, C., van de Locht, L., Tonnisen, E., Godsaid, F. and Raemaekers, J. (2001). A novel method to compensate for different amplification efficiencies between patient DNA samples in quantitative real-time PCR. Journal of Molecular Diagnostics, 3: 51-61. Doi: 10.1016/S1525-1578(10)60652-6.

Meredith, S.E.O., Lando, G., Gbakina, A.A., Zimmerman, P.A. and Unnasch, T.R. (1991). Application of Polymerase Chain Reaction to Identification of and Strain Differentiation of Parasite. *Experimental Parasitology*, 73:335-344.

Morales-Hojas, R., Post, R.J., Shelley, A.J., Maia-Herzog, M., Coscarón, S., Cheke, R.A. (2010). Characterization of Nuclear Ribosomal DNA Sequences from *Onchocerca volvulus* and *Mansonella ozzardi* (Nematoda: Filarioidea) and Development of a PCR-Based Method for their Detection in Skin Biopsies. *International Journal of Parasitology*, **31**:169-177.

Moya, L., Herrador, Z., Ta-Tang, T.H., Rubio, J.M., Perteguer, M.J., Hernandez-González, A., *et al*. (2016). Evidence for suppression of onchocerciasis transmission in Bioko Island, Equatorial Guinea. PLoS Negl Trop Dis 10(7): e0004829. doi:10.1371/journal.pntd.0004829.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., et al. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28: E63.

Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell Probes 16: 223–229.

Nagamine K, Watanabe K, Ohtsuka K, Hase T, Notomi T (2001) Loop mediated isothermal amplification reaction using a non-denatured template. Clin. Chem 47: 1742–1743.

Nutman, T.B., Parredes, W., Kubofcik, J. and Guderian, R.H. (1996). Polymerase chain reaction-based assessment after macrofilaricidal therapy in *Onchocerca volvulus* infection. *Journal of Infectious Diseases*, 173(3):773-776.

Osue, H. O., Inabo, H. I., Yakubu, S. E, Audu, P. A., Galadima, M., Odama, L. E., Musa, D., Ado, S. A. and Mamman, M. (2013). Impact of eighteen-year varied compliance to onchocerciasis treatment with ivermectin in sentinel savannah agrarian communities in Kaduna State of Nigeria. Hindawi Publishing Corporation *ISRN Parasitology*, 2013:1-10.

Osue, H. O., Inabo, H. I., Yakubu, S.E. and Audu, P. A. (2017). Comparing reliability of skin microfilarial load with first internal transcribed spacer primers for DNA amplification in monitoring onchocerciasis treatment control. *Nigerian Journal of Parasitology*, 38(1): 14-21.

Poole, C. B., Tanner, N. A., Zhang, Y., Evans, T. C. Jr. and Carlow, C. K. S. (2012) Diagnosis of Brugian Filariasis by Loop-Mediated Isothermal Amplification. PLoS Negl Trop Dis 6(12): e1948. doi:10.1371/journal.pntd.0001948

Powers, T.O., Todd, T.C., Burnell, A.M., Murray, P.C.B., Fleming, C.C., Szalanski, A. L., Adams, B.A. and Harris, T.S. (1997). The rDNA Internal Transcribed Spacer Region as a Taxonomic Marker for Nematodes. J*ournal of Nematology*, 29:441-450.

Robin ED, Wong R. (1988). Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J Cell Physiol. 136(3):507–13.

Rodriguez-Perez, M.A., Lilly, B.G., Dominguez-Vazquez, A., Segura-Arenas, R., Katholi, C.R. and Unnasch, T.R. (2004). Polymerase Chain Reaction Monitoring of Transmission of *Onchocerca volvulus* in Two Endemic States in Mexico. *American Journal Tropical Medicine and Hygiene*,70:38-45.

Scott, A. L., Dinman, J., Sussman, D. J., Yenbutr, P. and Ward, S. (1989). Major sperm protein genes from *Onchocerca volvulus*. *Molecular Biochemistry Parasitol*ogy, 36:119–26.

Ta Tang, T-H.T., López-Vélez, R., Lanza, M., Shelley, A.J., Rubio1, J.M. and Luz, S. L.B. (2010). Nested PCR to Detect and Distinguish the Sympatric Filarial Species *Onchocerca volvulus*, *Mansonella ozzardi* and *Mansonella perstans* in the Amazon Region. *Memorial Institute de Oswaldo Cruz*, 105(6): 823-828.

Thompson, J.D., Higgins, D.G., Gibson, T.J. (1994). ClustalW: Improving the Sensitivity of Progressive Multiple Sequence Alignment through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Research*, 22*:*4673–4680.

Unnasch, T. R. and Williams, S. A. (2000). The genomes of *Onchocerca volvulus*. *Internal Journal of Parasitology*, 30:543–52.

Vlaminck, J., Fischer, P.U. and Weil, G.J. (2015). Diagnostic tools for onchocerciasis elimination programs. *Trends in Parasitology*, 31(11):571–82.

World Health Organization (2016). Guidelines for Stopping Mass Drug Administration and Verifying Elimination of Human Onchocerciasis, WHO 2016 [Internet]. Available From:Http://Apps.Who.Int/Iris/ Bitstream/10665/204180/1/9789241510011_Eng.Pdf?Ua=1

Zeng, W. and Donelson, J.E. (1992). The actin genes of Onchocerca volvulus. Molecular Biochemistry and Parasitology, 55 (1-2), 207-216.

Zimmerman, P.A., Guderian, R.H., Araujo, E., Elson, L., Phadke, P., Kubofick, J. and Nutman, L. (1994). Polymerase Chain Reaction-Based Diagnosis of *Onchocerca volvulus* Infection: Improved Detection of Patients with Onchocerciasis. *Journal of Infectious Diseases*, 169:686-689