Molecular Identification of *Onchocerca* Species among Residents of Benue and Cross River States, Nigeria, Using Known Microsatellites and Mitochondrial DNA

Odama Richard Ikani, Agada Ali Samuel[1], Amechi Nmesomachi Joy[2], Kenechukwu Chibuike Onyekwelu[2], Akighir John[3], Ezeh Charles Ogbonna[2]

Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Calabar, Calabar, [1]Department of Medical Biochemistry, College of Health Sciences, Benue State University, Makurdi, [2]Department of Medical Biochemistry, College of Medicine, University of Nigeria, Enugu Campus, Enugu, [3]Department of Biochemistry, Pharmacological Biochemistry/Toxicological Unit, Federal University Wukari, Traba State, Nigeria

Abstract

Background: Human onchocerciasis is a neglected tropical disease caused by *Onchocerca volvulus* and is transmitted by the species *Simulium damnosum*. Currently, onchocerciasis is estimated to have infected over 37million people in tropical Africa, Yemen and Latin America, resulting in a debilitating eye and skin disease in more than 5million people with over 1.2 million cases of visual impairment or blindness. Also, an estimate of about 120 million people is at risk of contracting the disease due to the breeding habit of the vector. Community-directed ivermectin administration has greatly reduced the infection burden in different parts of the world but they are persistent cases of onchocerciasis infection in Benue and Cross River States, Nigeria due to the terrain, relapse/poor ivermectin coverage, and COVID-19 outbreak. **Aims and Objectives:** To investigate the prevalence of onchocerciasis in these localities, this study reported the use of mitochondrial DNA and microsatellite markers for molecular identification studies of *Onchocerca* species amongst residents of Benue and Cross River State, Nigeria. **Materials and Method:** Three hundred (300) patients from Benue and Cross River State, Nigeria, 150 patients each from both states were screened using SD Bioline onchocerciasis test strip from South Korea with batch no: 61ADE002B for the detection of IgG4 antibodies against Ov16 in onchocerciasis. 25 were positive from Cross River State and 20 from Benue state. Six (6) from each state were sent for DNA extraction and PCR amplification (L1-6=Benue State, L6-12= Cross River) using polymerase chain reaction (PCR) amplification of mitochondrial cytochrome C oxidase subunit I (COXI) genes using HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' primers and the primers of the respective microsatellites; (GT)AT(GT)AT(GT)10, (GT)GC(GT)10, $(CAG)_2(CAA)_{10}(CAG)$, and (GT)11TT(GT). **Results:** The results showed amplification of COX 1 subunit of the mtDNA of *onchocerca volvulus* at 344bp DNA sequence and amplification of (GT)AT(GT)AT(GT)10 microsatellites at 193bp, (GT)GC(GT)10 at 180bp, $(CAG)_2(CAA)_{10}(CAG)$ at 209bp, and (GT)11TT(GT) 195bp respectively. **Conclusion:** This study clearly showed that active transmission of human onchocerciasis infection is still ongoing in Wanikade and Igede communities of Benue and Cross River State as evident by the skin lesions and depigmentation presented by a 55 years old woman and the molecular parasitological evidence of the incidence *Onchocerca volvulus* using the parasites' genetic materials. Therefore, we recommend intensifying community-directed ivermectin intervention in these states.

Keywords: Benue and Cross River states, microsatellites, mitochondrial DNA, Nigeria, *Onchocerca species*

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Introduction

Onchocerciasis, as a serious neglected tropical disease (NTD), is caused by the filarial nematode *Onchocerca volvulus*, which can lead to blindness and chronic disability.[1,2] The filariae are a group of tissue-dwelling nematodes of vertebrates that are spread by blood-sucking arthropods, Onchocerca

Address for correspondence: Dr. Odama Richard Ikani, Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Calabar, Calabar, Nigeria.
E-mail: ikanirichard@yahoo.com

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volvulus.[2,3] Currently, onchocerciasis is estimated to have infected about 37million people in tropical Africa, Yemen, and Latin America,[3,4] resulting in a debilitating eye and skin disease in more than 5 million people,[3,5] with over 1.2 million cases of visual impairment or blindness.[2] Also, an estimate of about 120 million people is at risk of contracting the disease due to the breeding habit of the vector. The World Health Organization (WHO) recommends annual or biannual mass administration of ivermectin[2,6] as an eradication approach alongside vector control,[7,8] due to its significant human health and socioeconomic burden since the 1970s.[9-11] Clinical symptoms of onchocerciasis reflect the developmental stage of the parasites, and the immune response by the host and are highly variable. The clinical symptoms of onchocerciasis are often present after the L3 stage of the parasite matures into an adult worm between 9 months and 2 years and these symptoms include the formation of subcutaneous nodules (onchocercomata), generalized pruritus in early infection,[11] and skin rashes, extreme itching, eye cataract, itching of the eyes, light sensitivity, loss of skin elasticity, loss of vision [Figure 1][12,13] in severe cases and change of the skin pigmentation [Figure 2]. These occur as a result of the inflammatory response to dying microfilaria and Wolbachia antigen (Punctuate keratitis/Snowflake opacities),[12,13] corneal fibrosis or opacification, and other ocular manifestation such as iridocyclitis, glaucoma, choroiditis and optic atrophy.[11,14]

Over the years, the WHO recommends ivermectin or moxidectin as the drugs of choice for treating onchocerciasis due to its micro-filaricidal properties,[7,11,13,15] and it is administered at least once yearly for dosing interval of 3–12 months for a period of 10–12 years[7,16] followed by vector control as elimination strategies.

However, there is a limitation to the use of Ivermectin in areas where Onchocerca volvulus co-exists with loa loa as it can result in severe adverse neurological events[7,11,16,17] and the sub-optimal (or atypical) responses to Ivermectin,[18] as well as the effect of COVID-19 pandemic, has resulted in the resurgence of onchocerciasis in the rural part of Benue and Cross River State, Nigeria. Also, in these tropical African regions, this illness is diagnosed through clinical manifestation and microscopic examination of skin samples for the presence of microfilaria. The limitation with this technique is that there is a high incidence of false-negative as in most cases microfilaria are not present in the skin samples of infected patients under examination and this has impaired researchers from avidly quantifying the prevalence of this illness in several regions.

Therefore, this study exploited the advances in molecular diagnosis, making use of genetic materials (mitochondrial deoxyribonucleic acid [Mt DNA] and microsatellites) peculiar to the concerned organism to cut down the cases of false negatives, and also scaled up quantifying the prevalence of this illness in these regions. The molecular identification of this parasite in Benue and Cross River State of Nigeria using known microsatellites and mitochondrial deoxyribonucleic acid (DNA) is essential[3] as this will support future basic and translational onchocerciasis research with particular relevance to the ongoing onchocerciasis elimination program and boost efforts to intensify community-based ivermectin intervention in these localities.

Materials and Methods

Study area

Benue State; one of the North Central states in Nigeria (latitude 4˚N and14˚N and longitude 2˚ and 15˚E)[19] has a population of about 4,253,641 in the 2006 census, located at coordinate 7°20′N 8°45′E. It is inhabited predominantly by the Tiv, Idoma, Igede, and Etulo natives respectively with its capital in Makurdi. The territory was initially known as Munshi Province until 1918 when the name of its dominant geographical feature, the 'Benue River' was adopted. Cross River on the other hand is a state in South-South Nigeria that is located at coordinate 5°45′N 8°30′E,[20] bordering Cameroon to the east. Its capital is Calabar and its name is derived from the Cross River (Oyono), which passes through the state. English and French are the major foreign languages of the state, while Bekwarra, Bette people, Ejagham, and Efik[21] are major indigenous languages of this state. These states are made up of several streams which empty into these major rivers, making them a perfect breeding ground for black flies. After a careful literature review, Benue State and the Cross River State of Nigeria was chosen because of the high incidence of onchocerciasis in these states and these states are bounded by River Benue and Cross River State River Basin respectively and its tributaries which serve as a reservoir for the breeding of black flies which are the sole host of Onchocerca volvulus. According to Onekutu, *et al*., (2018)[22] in the community of Kuhe and Gube of Benue state of Nigeria, research on 546 persons showed a high prevalence of onchocerciasis at 61% and 71% respectively. Also, Opera, *et al*., (2006)[23] show that certain patients were blind due to onchocerciasis and were positive to onchocerciasis test in the lower Cross River basin.

Screening test and sample collection

Three hundred patients with varying skin lesions from Benue and Cross River States, Nigeria, were recruited for this study. 150 each from both states and were screened using SD Bioline onchocerciasis test strip [Figure 3][24] from South Korea with batch no: 61ADE002B for the detection of IgG4 antibodies against Ov16 in onchocerciasis. 25 were positive from Cross River state and 20 from Benue state. Six (6) from each state were sent for DNA extraction and PCR amplification (L1-6=Benue State, L6-12= Cross River).[24] The SD BIOLINE contains a membrane strip, which is coated with recombinant Ov16 capture antigen on the test line region. The anti-human IgG4-gold colloid conjugate and the specimen move along the membrane chromatographically to the test region (T) and form a visible line as the antigen-antibody gold particle complex is formed with a high degree of sensitivity and specificity.[24,25]

Both test and control windows are clearly labelled "T" and "C" respectively.

Mitochondrial deoxyribonucleic acid extraction

Whole-cell DNA was extracted from fresh blood samples frozen at -20°C after collection of the specimen using QIAGEN DNeasy Blood & Tissue kit from Southern Cross Biotechnology Ltd, South Africa.[25] Samples were first lysed using proteinase K with buffering conditions adjusted to provide optimal DNA binding, and the lysate is loaded onto the DNeasy Mini spin column and centrifuged. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors are removed in two efficient wash steps and DNA is then eluted in the buffer ready for use.

Polymerase chain reaction amplification

Onchocerciasis infection identification was done using PCR amplification of mitochondrial cytochrome C oxidase subunit I (COXI) genes using HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' primers. 25 µl reaction mixtures contained 2.5 µl of Super Taq 10x buffer, 0.5 µl of HC02198 forward primer, 0.5 µl of HC02198 reverse primer, 0.2 µl of 100 mM dNTP, 1 µl of DNA polymerase, 14.55 µl of distilled water, 0.75 µl of $MgCl_2$, and 5 µl of sample DNA. The PCR condition involved an initial denaturation step at 95°C for 2 min, followed by 37 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension step at 72°C for 5 min.[25] The expected band size is 344 bp.

Amplification of *Onchocerca* microsatellites [Table 1] was done using PCR-based microsatellites' respective primers. All amplifications were performed using 25 µl reaction mixtures containing 2.5 µl of Super Taq 10x buffer, 0.5 µl of the forward primer, 0.5 µl of the reverse primer, respectively, 0.2 µl of 100 mM dNTP, 1 µl of DNA polymerase, 14.55 µl of distilled water, 0.75 µl of $MgCl_2$, and 5 µl of sample DNA. The PCR condition involved an initial denaturation step at 95°C for 2 min, followed by 37 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s with a final extension step at 72°C for 5 min. Then, 5 µl of PCR product each was mixed with standard loading dye and electrophoresed alongside 50 bp DNA ladder in 1.5% agarose stained with ethidium bromide (0.5 µg/ml). The products were visualized and photographed under ultraviolet illumination.

Table 1: Onchocerca microsatellites, primer sequence, and amplified band size

Microsatellites	Primer sequence	Band sizes (bp)
(GT)AT(GT) AT$(GT)_{10}$	CCCATTTGCCAGTTGAGGTGA-F CCCGTCAACATTGTGGCTACG-R	193
(GT)GC$(GT)_{10}$	GACGCATACCGAGTCCTTGT-F TACGCACACATTTTTCTATTTC-R	180
$(GT)_{11}$TT(GT)	CGCTAACGCTGTGCAATATTG-F TGACGAACTTTGGGACGACA-R	195
$(CAG)_2(CAA)_{10}$ (CAG)	CGACAACGTGTCTCGACAAA-F CGAAAACAACATACGAAGGG-R	209

Results

Twenty-five people were positive from Cross River state and 20 from Benue state using the onchocerciasis SD BIOLINE test strip. Six samples from each state were subjected to PCR amplification (L1–6 = Benue state and L 6–12 = Cross River).

On visualization, the results show amplification of COX 1 subunit of the mtDNA of onchocerca volvulus across L1-12 using PCR *HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3 9* [Figure 4].

Marked amplification of (GT)AT(GT)AT(GT)10 microsatellite across sample L1, L2, L3, L5, and L6 from Benue and sample L7, L8 and L11 from Cross River State using CCCATTTGCCAGTTGAGGTGA-F, CCCGTCAACATTGTGGCTACG-R microsatellites primer sequence were observed.

Furthermore, there was an amplification of (GT) GC$(GT)_{10}$ microsatellite in the sample L3 and L5 from Benue State and sample L12 from Cross River State using GACGCATACCGAGTCCTTGT-F, TACGCACACATTTTTCTATTTC-R microsatellite sequence [Figure 5], as well as amplification of (CAG) $_2$(CAA) $_{10}$(CAG) *O. volvulus* microsatellites in the sample L1–L6 from Benue and L7, L9, L10, and L12 from Cross River state using CGACAACGTGTCTCGACAAA-F; CGAAAACAACATACGAAGGG-R microsatellite sequence [Figure 6] followed by amplification of (GT) $_{11}$TT (GT) *O. volvulus* microsatellites in sample L3, L5, and L6 from Benue and L7–L12 from Cross River states using CGCTAACGCTGTGCAATATTG-F, TGACGAACTTTGGGACGACA-R microsatellite sequence, respectively [Figure 7].

Figure 1: Loss of vision[12,13]

Discussion

Over the years, microsatellites and Mt DNA are regarded as molecular tools[26] for species identification and characterization, diagnostic target for infectious diseases, and estimation of divergence on evolutionary timescales and have become essential in many areas of evolutionary biology, including system biology, biochemistry, and molecular biology.[26] Therefore, in this study, we employed microsatellites and Mt DNA to carry out molecular identification of *Onchocerca* species among patients from Benue state and Cross River state of Nigeria to extrapolate the prevalence of onchocerciasis infection on the molecular level.

The PCR primers HC02198 (5' TAAACTTCAGGGT GACCAAAAAATCA 3') [Figure 4], and microsatellite primers (5'-CCCATTTGCCAGTTGAGGTGA-3'-F, 5'-CCCGTCAACATTGTGGCTACG-3'-R; 5'-GACGCA TACCGAGTCCTTGT-3'-F, 5'-TACGCACACATTT TTCTATTTC-3'-R; 5'-CGCTAACGCTGTGCA ATATTG-3'-F, 5'-TGACGAACTTTGGGACGACA-3'-R; 5'-CGACAACGTGTCTCGACAAA-3'-F; 5'-CGAAAACAACATACGAAGGG-3'-R) were designed to amplify the COX 1 portion of *O. volvulus* Mt DNA (GenBank accession number AF015193)[27] and (GT) AT (GT) AT (GT) $_{10}$ [Figure 8], (GT) GC (GT) $_{10}$ [Figure 6], (CAG) $_{2}$(CAA) $_{10}$(CAG) [Figure 6], (GT) $_{11}$TT (GT) [Figure 7] microsatellite portions, respectively, of *O. volvulus* (GenBank accession number AF015193)[27] for identification of *O. volvulus* among onchocerciasis-infected patients. Primer size for both forward and reverse primers were 50bp each with an annealing temperature of 55°C.Visualisation of the gel image performed on the obtained sequences showed amplification of 344bp DNA sequence (EMBL GenBank accession: (*O. volvulus* AF228575, AF228574, AF228565))[27,28] which codes for *O. volvulus* COX subunit 1 gene (GenBank accession number accession HG738213.2)[26] and 193bp, 180bp, 209bp and 195bp microsatellites DNA sequence respectively[29] (EMBL GenBank accession: *O. volvulus* AF228565)[26,27] coding *O. volvulus* microsatellite portions. (GenBank accession number accession HG738213.2)[30]

Three hundred patients examined presented with varying features of onchocerciasis as seen in the case of a 55-year-old woman from Barracks-Wanikade, North Ukelle [Figure 2], with skin lesion and depigmentation, which shows evidence of ongoing incidence of onchocerciasis in these localities. The result of this study showed 30% prevalence rate in Benue and 37% Cross River prevalence when expressed against the 150 patients from each state. Cases of infection tend to be higher among people of this community because these are riverine areas and favor the breeding of black flies.[30] This agrees with the studies reported in different parts of Nigeria by Oyibo & Fagbenro, (2003)[31,32] (40%) on the Effect of repeated community-based Ivermectin treatment on the intensity of onchocerciasis in Nigeria; Adesina, *et al*.,(2017)[33] on the Incidence of onchocerciasis and vector knowledge among

Figure 2: A 55-year-old woman from Wanikade, North Ukelle, of Cross River state of Nigeria with skin lesion and depigmentation

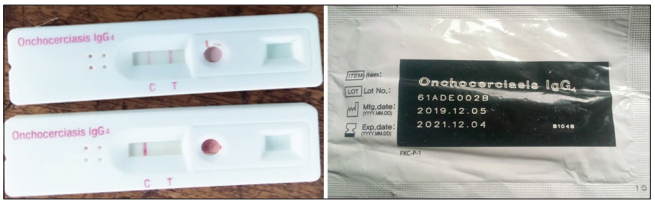

Figure 3: SD BIOLINE onchocerciasis IgG4 (61FK10) rapid test strip[24]

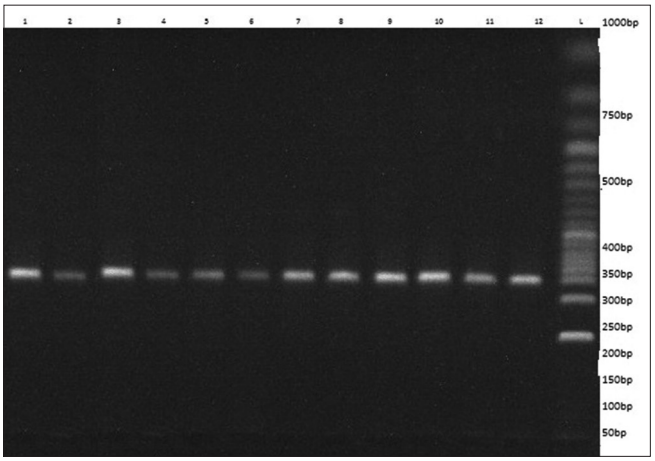

Figure 4: A representative agarose gel picture showing the resolution of the amplified products of COX1 using HC02198: 5'-TAAACTTCAGGGTGACCAAAAAATCA-3'

residents of some part of Ondo State, Nigeria (75%); Onekutu, *et al*.,(2018)[22] on the prevalence and distribution of human onchocerciasis and dermatological features in Kwande Local Government Area of Benue State, Nigeria (61%–71%).

Further, all the samples that were positive using Standard BIOLINE Onchocerciasis test strips were subjected to PCR amplification of *O. volvulus* Mt DNA COX1 gene subunit and microsatellites. This showed marked amplification across the samples which ascertain that onchocerciasis infection remains a serious neglected tropical disease of public health concern in

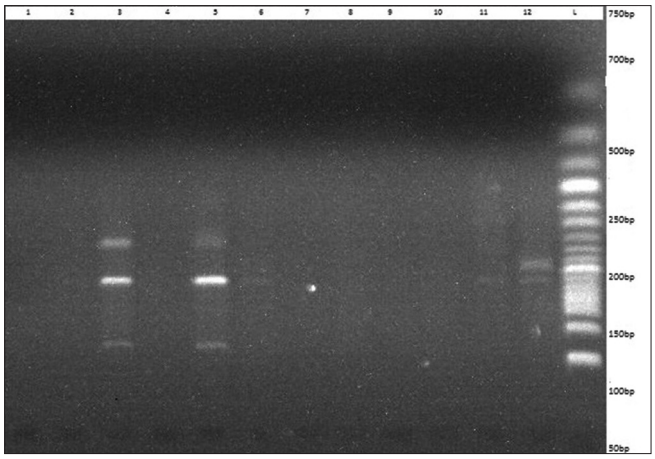

Figure 5: A representative agarose gel picture showing the resolution of the amplified products of (GT) GC (GT) 10 microsatellite using GACGCATACCGAGTCCTTGT-F TACGCACACATTTTTCTATTTC-R in 5'-3' direction

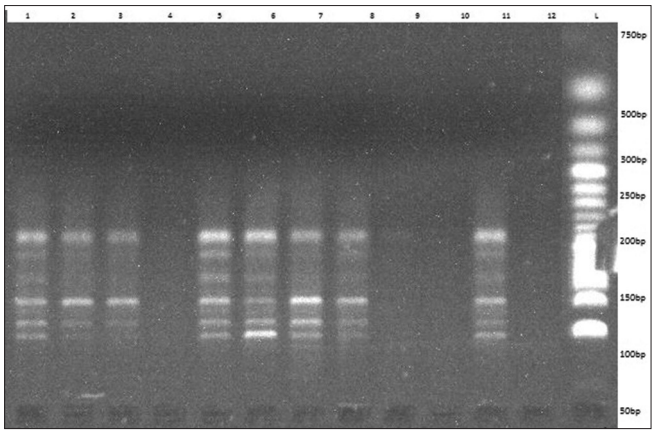

Figure 8: A representative agarose gel picture showing the resolution of the amplified products of (GT) AT (GT) AT (GT) 10 microsatellite using CCCATTTGCCAGTTGAGGTGA-F CCCGTCAACATTGTGGCTACG-R in 5'-3' direction

Benue and Cross River state of Nigeria. This established a nexus between the persistent ongoing transmission of onchocerciasis infection in these states in Nigeria and the continuous exposure to black flies in these localities as confirmed by marked amplification of *O. volvulus* microsatellites and Mt DNA from infected patients. This shows that though the burden of onchocerciasis is reduced in some region of the country due to community-based ivermectin distribution, it is still of health concern in riverine areas of Benue and Cross River states of Nigeria. This is in agreement with a study carried out in Ghana which showed that despite the community-directed ivermectin treatment, the prevalence of *O. volvulus* infection still remains a health concern in different parts of the tropics.[2,28,34]

Conclusion

Although the burden of onchocerciasis is reduced in different parts of the world, this study clearly showed that active

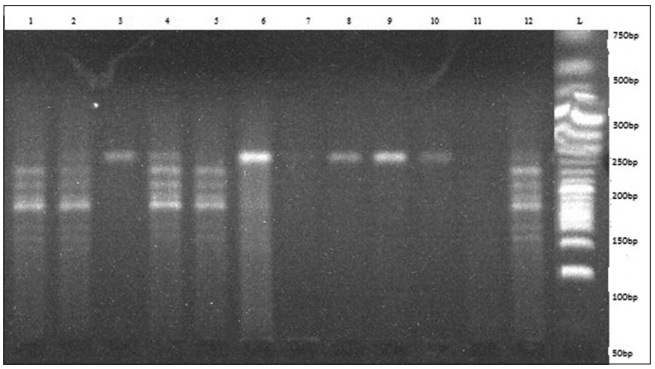

Figure 6: A representative agarose gel picture showing the resolution of the amplified products of $(CAG)_2(CAA)_{10}(CAG)$ microsatellite

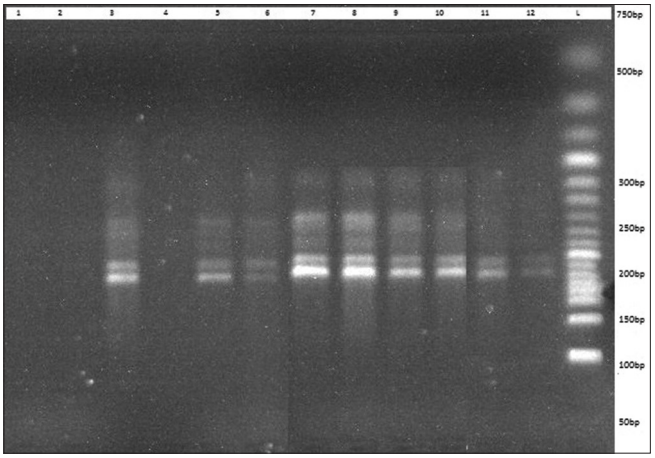

Figure 7: A representative agarose gel picture showing the resolution of the amplified products of $(GT)_{11}TT$ (GT) microsatellite

transmission of human onchocerciasis infection is still on going in Wanikade and Igede communities of Benue and Cross River States as evident by the skin lesions and depigmentation presented by a 55-year-old woman and the molecular parasitological evidence of the incidence *O. volvulus* using the parasites' genetic materials. Therefore, we recommend intensifying community-directed ivermectin intervention in these states.

Ethical approval

Ethical approval for this study was obtained from Research and Ethics Committee of College of Medicine, University of Nigeria, with ethical clearance notification. Protocol No: 082/12/2019 and Government of Benue State of Nigeria with Ref. MOH/OFF340/VOL/4P8912.

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Declaration of patient consent

The authors certify that they have obtained all appropriate patient consent forms. In the form the patient(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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