Detection and Discrimination of *O. volvulus* and *O. ochengi* from Blackfly Pool DNA using a Novel Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) Technique.

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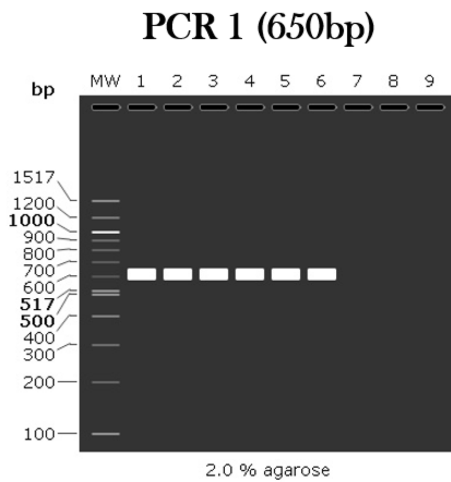
Molecular xeno-monitoring has been a significant technique used to study the impact of vector-borne pathogenic diseases on humans and animals by employing disease surveillance in vector populations. It uses insects carrying pathogen genetic material as a non-invasive surrogate for infection in the human or animal population. In the case of onchocerciasis, the WHO has approved the O150 PCR in blackflies, a molecular xenomonitoring technique, as part of the Onchocerciasis Elimination guideline. However, this technique is laborious and time-consuming, which could be influenced by human errors. Also, the high cost, non-availability, and delay of the ELISA component make the application of the O150 PCR difficult in resource-limited settings. Therefore, this study focused on developing a PCR-RFLP assay to detect and discriminate *O. volvulus* and *O. ochengi* in blackflies.

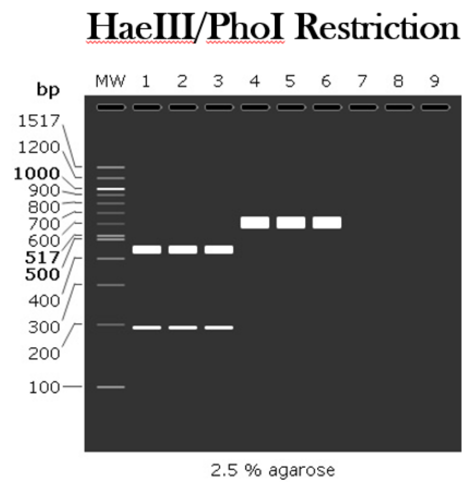
Bioinformatics analysis was employed to identify a unique restriction site within the *COX1* mitochondrial gene sequences of *O. volvulus* and *O. ochengi*. HaeIII, unique to *O. volvulus* only, was identified as the restriction enzyme of choice for the discrimination. Onchocerca-genus primers were designed in the conserved regions of both *O. volvulus* and *O. ochengi* sequences to amplify a 650 bp fragment, which flunks the restriction site. From the conserved sequences, *Onchocerca-COX1* probe was also designed to be used in the magnetic beads capture of Onchocerca-DNA from blackflies DNA pool. Assay validation was done with *Onchocerca sp.* sequence data retrieved from the NCBI Genbank. The wet-lab validation of this assay was performed with archived blackflies (*S. damnosum sensu lato*) collected in 2011 from Agbelekeme, an endemic onchocerciasis community. Triplicates of 50 and 100 Blackfly pools were performed separately for heads and bodies. Blackfly pool DNA was extracted, and the Onchocerca-DNA was captured with the *Onchocerca-COX1* probe and magnetic beads. The PCR-RFLP assay was applied to the Onchocerca-captured samples, after which the PCR products were sequenced and analyzed.

The restriction site, GG|CC (HaeIII), was unique to only *COX1 O. volvulus* sequences in the NCBI GenBank since they produced 456 bp and 194 bp fragments from the 650 bp PCR product. Of the three 50 blackfly head pools, only 1 (1/3) carried infective *O. ochengi* larval stage. All three 100 blackfly head pools (3/3) carried infective *O. volvulus* larval stage. However, two of the three 100 blackfly body pools (2/3) were infected with *O. ochengi*. Only one of the two infected 50 blackfly body pools carried both *O. volvulus* and *O. ochengi*. The PCR amplicons with the restriction sites showed high homology with *O. volvulus*, whereas the unrestricted amplicons were highly homologous to *O. ochengi* after the DNA sequence analysis.

The novel PCR-RFLP assay has demonstrated its effectiveness in detecting and discriminating between *O. volvulus* and *O. ochengi* in blackflies. In addition, with zoonotic onchocerciasis in sight in some parts of the world, this tool will be useful for the early detection of potential zoonotic transmission of the bovine onchocerciasis in the human population, especially in Sub-Sahara Africa.

ASSAY DESIGN (GEL SIMULATION)





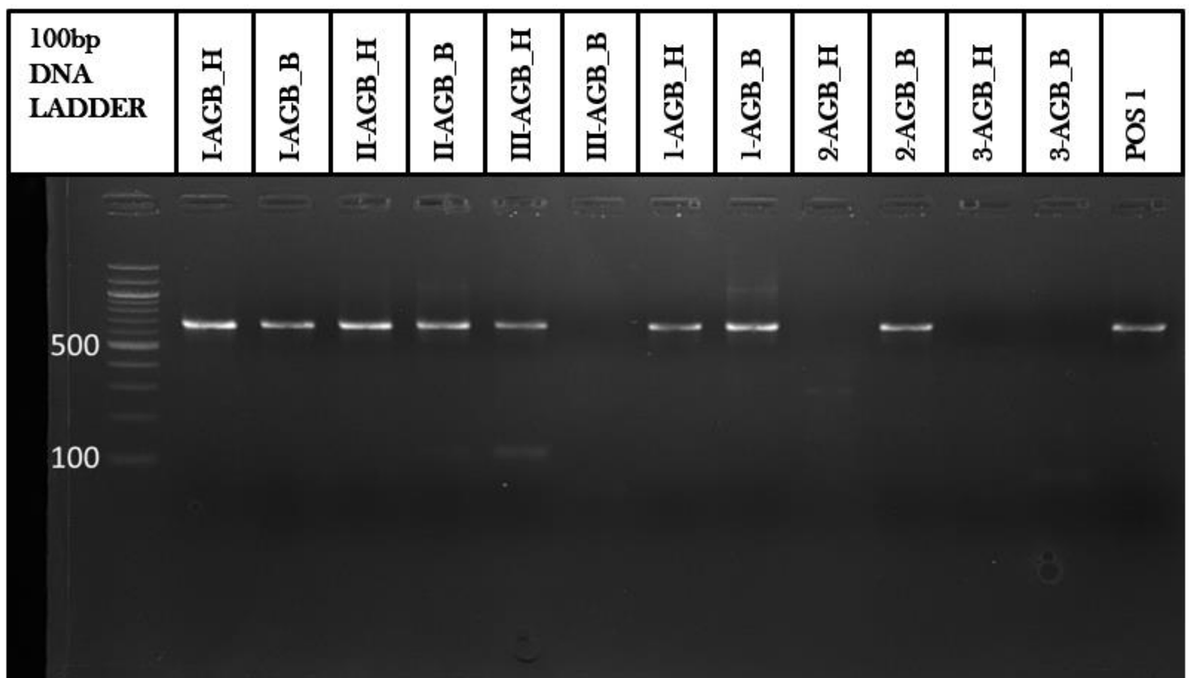
NOTE

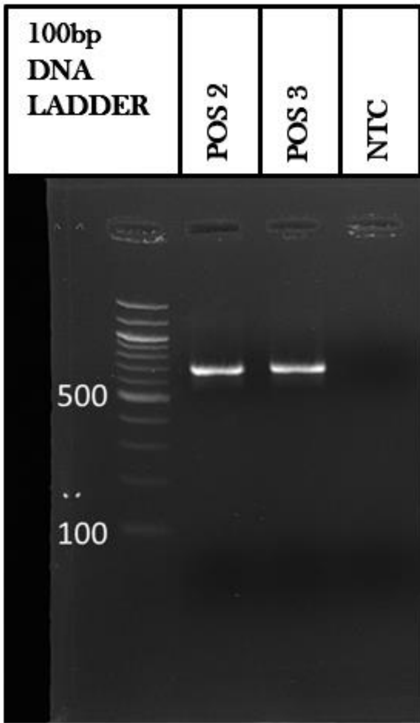
Sample 1, 2 &3: *O. volvulus*

Sample 4, 5 &6: *O. ochengi*

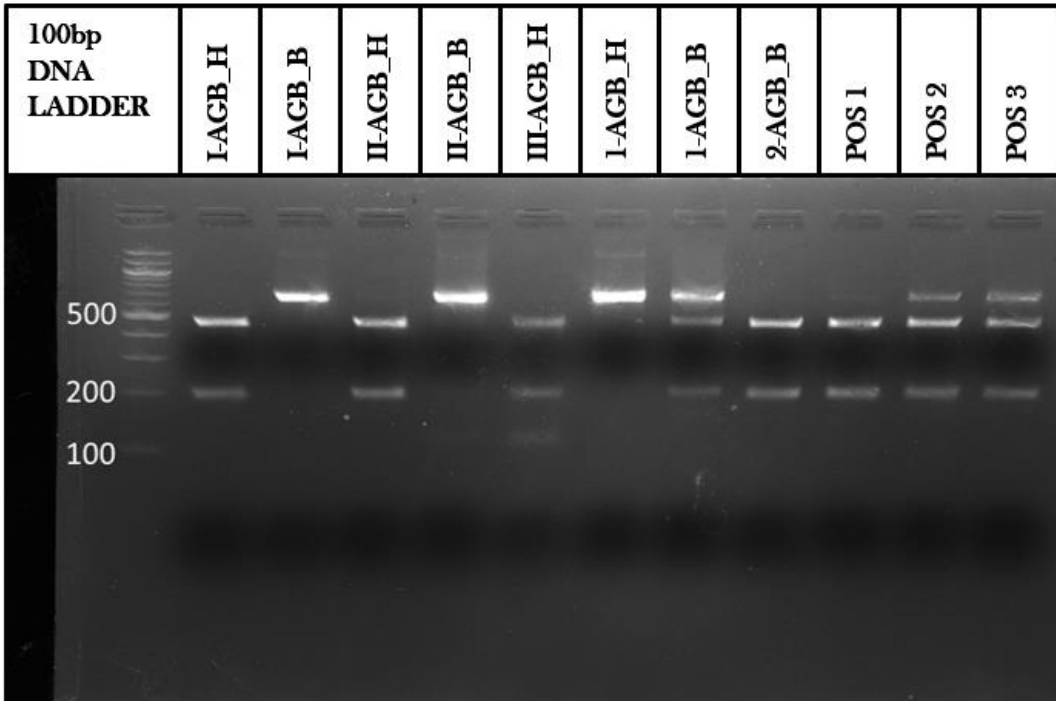
WET LAB CONFIRMATION

- **Onchocerca genus PCR**

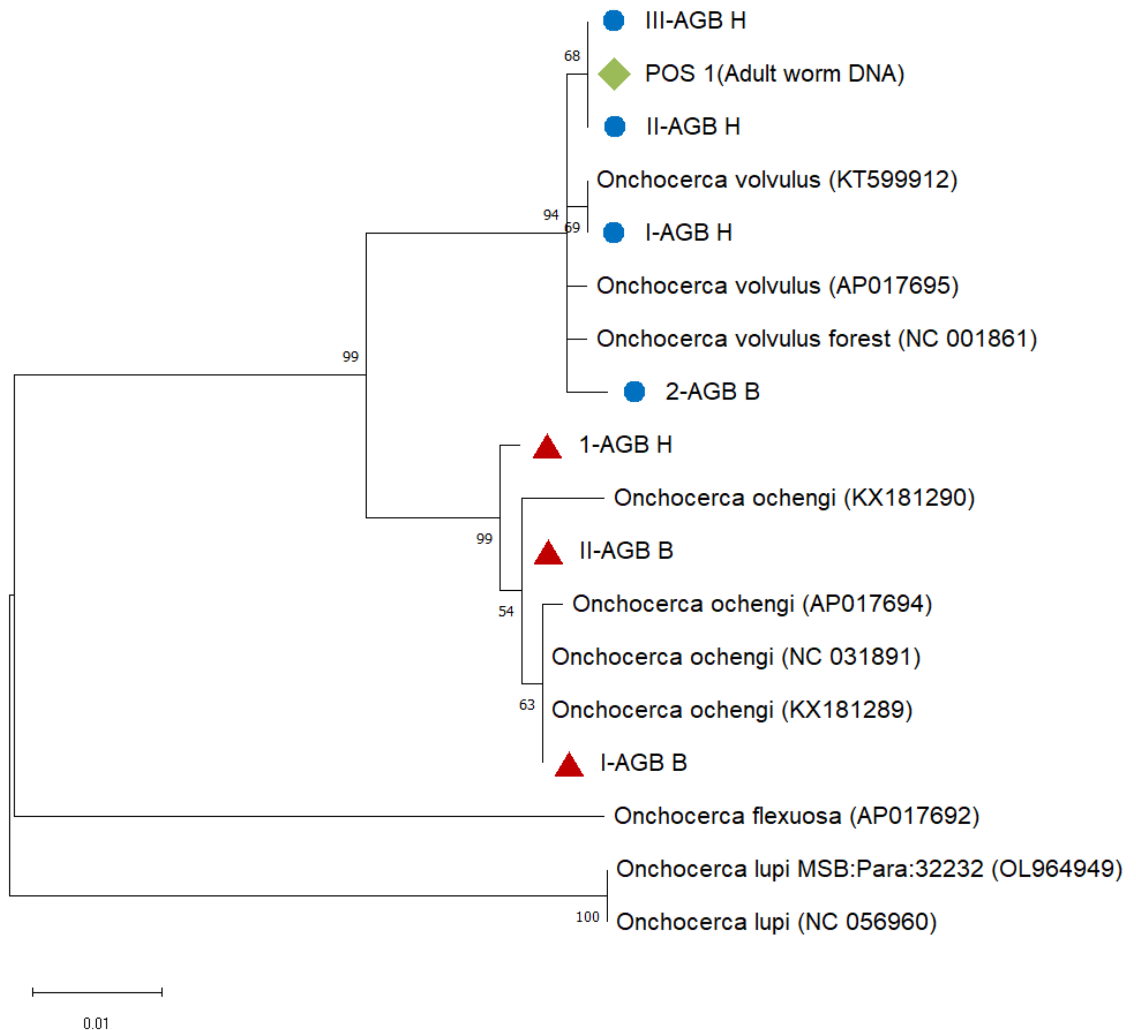




- **Onchocerca species Discrimination Restriction Fragment Length Polymorphism (HaeIII)**



- **Phylogenetic analysis (DNA Sequence analysis)**



Evolutionary analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model [1]. The tree with the highest log likelihood (-1446.34) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 18 nucleotide sequences. There were a total of 631 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 [2]

1. Kimura M. (**1980**). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**:111-120.
2. Tamura K., Stecher G., and Kumar S. (**2021**). MEGA 11: Molecular Evolutionary Genetics Analysis Version 11. *Molecular Biology and Evolution* https://doi.org/10.1093/molbev/msab120.