Identification of the forest strain of *Onchocerca volvulus* using the polymerase chain reaction technique

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Summary

Annual mass treatment with ivermectin for 12 – 15 years in endemic communities is the control strategy adopted by the African Programme for Onchocerciasis Control (APOC) for the control of onchocerciasis in Nigeria. This long-term treatment necessitates the use of Polymerase Chain Reaction (PCR) for the proper identification of the *Onchocerca species* and strains in endemic areas and also for monitoring recrudescence of infection in areas where infection has been controlled. This study, which forms part of a larger study on transmission of onchocerciasis identifies the *Onchocerca volvulus* strain in Ondo state using the Polymerase Chain Reaction (PCR) technique.

Deoxyribonucleic acid (DNA) was extracted from the adult worm of *Onchocerca* parasite using the glass bead method of extraction. The repeated sequence family present in the genome of the parasite designated as 0-150bp was amplified by the polymerase chain reaction (PCR).

The amplified parasites produced significant products visible as bands in a 2% agarose gel stained with ethidium bromide. Hybridization of the PCR products with specific DNA probe identified the products as forest strain of *Onchocerca volvulus*. The epidemiological implication of this is that there would be more of the skin lesions and low blindness rate in the area.

Keywords: ***Onchocerca volvulus, Identification, Polymerase chain reaction.***

Résumé

Traitement du masse annuel avec Ivermectin pour 12 - 15 ans des communautés endémiques est la stratégie de contrôle adoptée par le Programme Africain pour le Contrôl de l'onchocercose au Nigeria. Le traitement de longue durée rend nécessaire l'utilisation de Polymerase Réction en Chaine (PRC) pour une bonne identification des espèces d'onchocercose et entorse dans les zones endémiques et aussi pour la surveillance de la recrudescence de l'infection dans les régions où on avait controllé l'infection. Cette étude, qui fait partie d'une étude plus approfondie de la transmission d'onchocercose, identifie l'entorse volvulus onchocerca dans l'Etat d'Ondo avec l'utilisation de la méthode de la polymerase Réaction en Chaine (PRC).

Acide Deoxyribonucleigne (DNA) était arraché des vers adultes du parasite onchocerca avec l'utilisation de la méthode d'extraction du glass bead. L'ordre de famille présente répété dans le génome du parasite désigné comme 0 - 15 obp était amplifié par le polymerase réaction en chaine (PRC).

Les parasites amplifiés avaient constitué un produit important visible comme bands dans un 2% agarose gel entorse avec ethidium bromure. L'hybridation des produits de PCT avec la sonde DNA spécifique a identifiè les produits comme l'entore foret de volvulus onchocerca de la forêt. L'implication épidémiologique de ceci est qu'il aura encore des lésions de la peau et le taux bas de la cécité dans la région.

Introduction

Many lines of evidence have suggested that at least two strains of *O. volvulus* exist in West Africa [1,2,3,4]. These "forms" differ markedly in their transmittability by *Simulium* vectors, their infectivity to chimpanzees, their general epidemiology, and the severity of clinical expression [5]. The savannah strain, found in West African savannah, is capable of inducing ocular pathology in a large proportion of individuals it infects [6]. The forest strain, endemic in the West African rain forest, appears to be less able to induce ocular disease, even in individuals with high parasite load [2]. In the transition zone between the savannah and the forest an intermediate disease pattern exists, in which blindness rates are halfway between the savannah and the forest [7]. This pattern has been attributed to the coexistence of savannah and forest strains of parasites in these areas [8]. In the past years, three groups have reported progress towards development of an assay using DNA probes [9, 10, 11]. In each case, the DNA sequences isolated from *O. volvulus* appear to show slightly differing degrees of cross-reactivity with genomic DNAs from other filarial species. The relationship between these sequences, if any, remains to be established [12]. Zimmerman et al. [13] screened the genomic libraries and developed oligonucleotide probes based upon repeated sequence families. The repeated sequence family present in the genome of *Onchocerca* parasites, designated O-150, was amplified from various samples of genomic DNA using PCR. The DNA sequence analysis of the resulting PCR products demonstrated that the sequences may be arranged into clusters within which the individual sequences are identical or nearly identical. Differences among the cluster consensus sequences have been exploited to explain the specificities of previously isolated O-150 based probes and to develop two new oligonucleotide probes. One of these probes hybridizes specifically to *Onchocerca volvulus* O-150 PCR products, while the second hybridizes specifically to O-150 PCR products from the closely related bovine parasite *O.ochengi.* These oligonucleotide probes have been used to characterize *Onchocerca* infective larvae isolated from wild caught infected flies in West Africa.

The precise identification of the species transmitting a disease is fundamental to the epidemiology of the disease, there is therefore a need for DNA-based identification of *Onchocerca* species. This will not only be useful in

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differentiating between two morphologically similar *Onchocerca* species but also for monitoring transmission and detection of recrudescence of *O. volvulus* following mectizan treatment in endemic communities by screening *Simulium* in large batches since in most areas only a fraction of the vector are infected. Furthermore, it enables the differentiation of the different strains of the parasite, which is of great importance to the control programme. This present study identifies the forest strain of *Onchocerca volvulus* using Polymerase Chain Reaction (PCR) based techniques.

Materials and method

Ethical consideration

Prior to the commencement of the study, approval for the study protocol was obtained from the ethics committee of the Nigerian Institute of Medical Research (NIMR). Similarly approvals for the study were obtained at the State Ministry of Health, Local Government, and Community levels. At the micro level the informed consent of the respondents was obtained before they were recruited to participate in the study. The respondents were made to know the benefits and discomfort of their participation in the study. Patients with nodules were educated on the pathological effects of nodules in their bodies and were given the opportunity for nodulectomy at the General Hospital Akure at the expense of the project. Patients that consented to nodulectomy were taken to the General Hospital Akure. Nodulectomy was performed by a qualified Medical Doctor. Expenses of nodulectomy was borne by the project and for a period of one week that the patients were asked to rest at home, a subsistence allowance equivalent to their one week income was provided.

Parasite identification

Recovery of parasite samples

Onchocercomata were surgically removed from infected humans in Owena community in Ondo state and preserved in isopropanol. The adult parasites were eventually dissected out from the surrounding host tissue. These and the microfilariae that emerged from skin snip were separately preserved in isopropanol.

DNA extraction

The parasite was transferred to an eppendorf tube containing 25μl Tris EDTA (TE). Following standard proceedure [14] the parasite was treated with 5μl proteinase K at 56°c, 10μl of 1M Dithiothreitol (DTT) and 460μl of TrisEDTA (pH8.0) and boiled at 100°c for 30mins. The aliquot was further subjected to three cycles of freezing and thawing in isopropanol chamber at -70°c to release the genomic DNA. This was purified in ethanol wash using Salmon sperm DNA as the carrier in the presence of high salt concentration (NaI) and glass slurry. Parasite DNA was eluted from the glass slurry into 100μl of TE.

Polymerase Chain Reaction (PCR) amplification

The *Onchocerca* repeated sequence designated 0-150 was amplified in a solution containing 100mM tris-HCl (pH 8.3), 500mM KCl, 15mM $MgCl_2$, 0.1% gelatin, 2mMdNTP, 20mM of each of the two amplification primers, 2.5units of Taq DNA polymerase and 5μl of purified genomic DNA. The reactions were subjected to 35 cycles of amplification with each cycle consisting of 1 minute at 94°c, 2 minutes at 37°c and 30secs. at 72°c.

Agarose gel electrophoresis

Electrophoresis was carried out in a 2% agarose gel (Le agarose SEKEM FMC). 45μl of PCR products was transferred into a clean microfuge tube and mixed with 5μl of loading buffer (Bromophenol blue). The gel tank was connected to a 150v power source for 1 hour. Following electrophoresis, the gel was stained with 10% ethidium bromide in TAE buffer and visualised on Ultra-violet trans-illuminator. Polaroid photographs of ethidium stained gel were taken and bands of PCR positive samples identified and recorded on PCR work sheet.

Southern blot

The gel was denatured for 30minutes in denaturing solution and neutralised for 15 minutes twice before its transfer from the agarose gel to a hybond bond filter by capillary action in a high salt buffer (Saline Sodium Citrate (SSC). The arrangement was left overnight. The transferred DNA was permanently bonded to the filter by baking in Ultra-violet strata-linker.

Hybridization

Stored filters were first treated with 10ml of pre-hybridization buffer in sealed plastic bags at 42°c for at least 2 hours, followed with hybridization buffer containing 30μl of labelled probe and incubated at 42°c overnight. Following hybridization filters were washed at specific condition to remove excess probe [14].

Immunological detection

Hybridized filters were washed for 2 min. in buffer 1 and incubated for 1 hour in buffer 2 and then washed again in Buffer 1. The filters were then treated with 10μl of the stock anti-digoxygenin antibody conjugate to detect the digoxygenin labelled probe with the complementary DNA sequence. The unbound antibody conjugate was then removed by washing twice for 15 min. in buffer 1. Filters were equilibrated for 2minutes in buffer 3 and incubated at room temperature with 10ml colour solution in sealed plastic bag until bands appeared. Reaction was stopped by washing twice in buffer 1 and dried at room temperature.

Results

The amplification of the PCR products is as shown in plate 1. On lane 1 is the positive control of the genus *Onchocerca,* while on lanes 2 - 4 are the DNA samples of the adult worm. On lanes 5 - 7 are the DNA sample of the microfilariae. On lanes 8 and 9 are the positive controls of the Forest and Savannah strain specific samples. Lane 10 is the negative control (-DNA).

The visible bands in the agarose gel at the end of the electrophoresis shows amplification of the O-150bp repeats of the genus *Onchocerca*, initiated by the 5^1 and 3^1 primers.

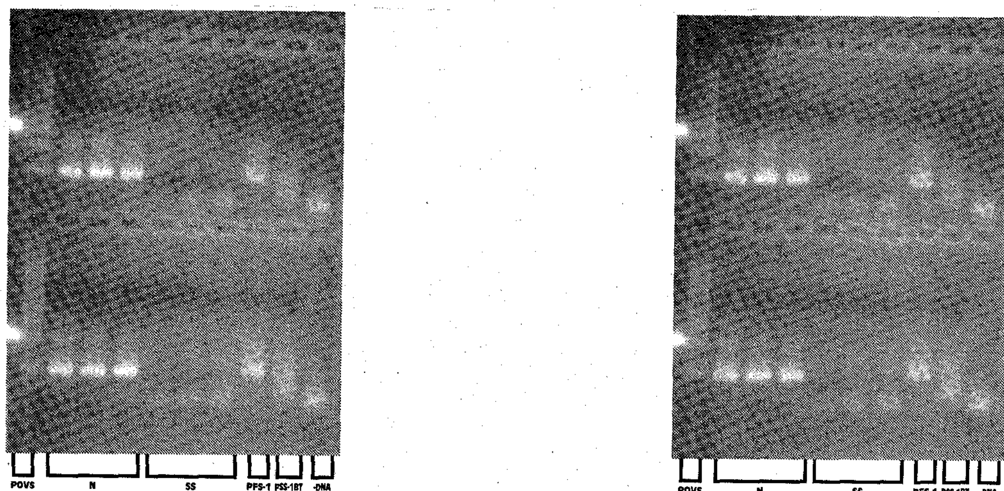

Fig. 1 ***Polaroid photographs of the Agarose gel electrophoresis***

POVS - *Positive Onchocerca control*
N - *DNA from adult worms*
SS - *DNA from Microfilaria*
PFS-1 - *Positive Control for Forest Probe*
PSS-IBT- *Positive Control for Savannah Probe*
-DNA - *Negative Control*

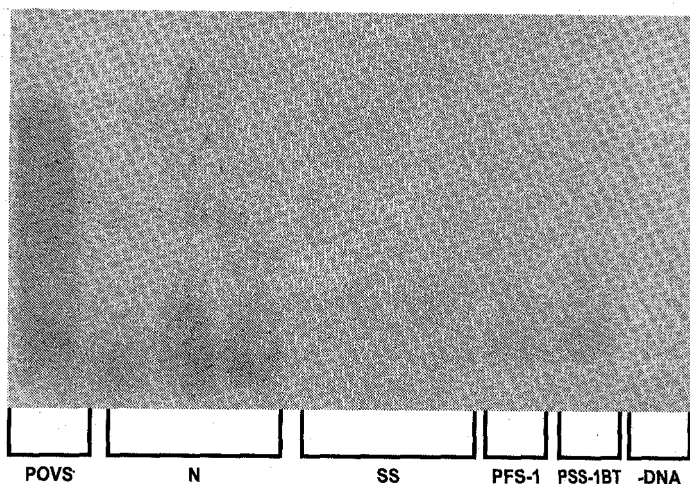

Fig. 2 ***Blot hybridized with OVS - 2***

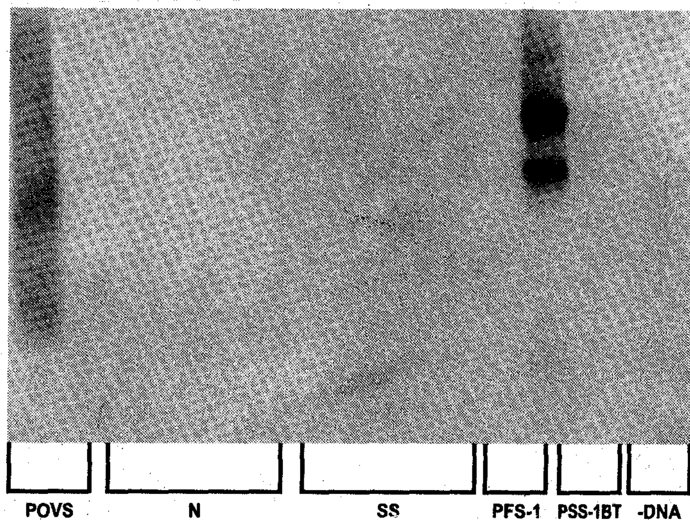

Fig. 3 ***Blot hybridized with probe (PFS - 1)***

This confirms that the PCR products belong to the genus *Onchocerca.* Hybridization of the PCR positive sample with species specific probe (OVS-2) identify the products as *Onchocerca volvulus* (Plate 2). There was positive signal with the forest strain specific probe when hybridized with the PCR product of the DNA sample (Plate 3), while the savannah strain specific probe was negative (Plate 4).

Discussion

The amplification of the target DNA from the Nigerian sample, using the primer of the tandemly repeated DNA sequence family found in the Onchocerca genome designated as 0-150 [13] confirms the kinship of the Nigerian *Onchocerca spp* with this consensus sequence. Its hybridization to OVS-2 confirms it to be *Onchocerca volvulus.* The hybridization of the sample to the forest probe confirms it to be forest strain of the parasite. Though, Awolola et al.[15] characterised the savannah strain of the parasite from Kainji in Niger state of Nigeria and observed that sample from the savannah region of the country did not hybridize with the forest or the savannah probe and this according to Toe (Pers. Comm) the savannah probe can only capture about 30% of the savannah parasite and the non hybridization of parasite to both forest and savannah probe signifies savannah strain. This has earlier been attributed to increase specificity and concomitant loss of sensitvity [12, 6].

The presence of the forest strain in this forest zone confirms the stability of the strain being transmitted. Though, Bissan et al. [17] noted seasonal influx of the savannah flies into some forest areas where there is no vector control in the Republic of Benin. This supports the observation of Toe et al. [18], that the forest dwelling flies can be efficient vector of the savannah strain of the parasite. Toe et al. [18] also stated that the coexistence of the savannah and forest strains of the parasite in the same area would have the resultant effect of the manifestation of an intermediate disease pattern in which blindness rate would

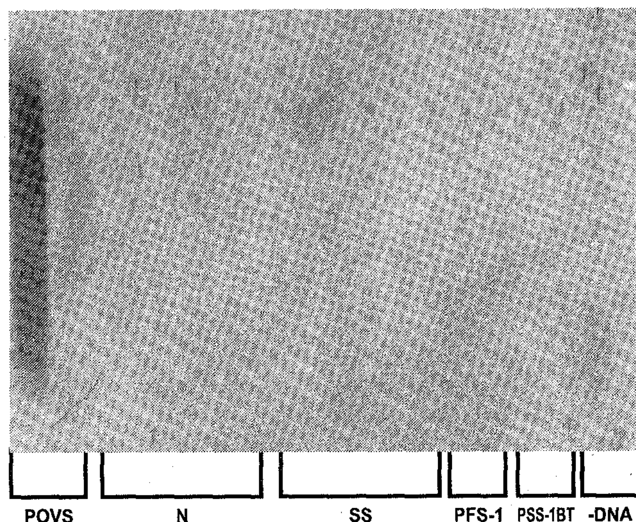

Fig. 4 *Blot hybridized with Savannah probe (PSS-1BT)*

be half way between those of the classical savannah and forest [19,7]. The confirmation of the forest strain of the parasite in this zone buttresses the very low prevalence of blindness observed in the area [20].

There is the need for further studies to elucidate more on the proper identification of the sibling species of *Simulium damnosum s.l* so as to establish the form of vector-parasite relationship that exists in the area. The application of this method in this study, enabled the differentiation of parasite strain, and thus buttressed the low prevalence of blindness and high level of skin lesion observed in the study area. Routine application of this method will enable transmission of infection to be monitored in areas of control.

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