Genomic fingerprinting of Onchocerca species using random amplified polymorphic DNA

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Abstract

A method based on amplification of genomic DNA by decamer primers of random nucleotide sequence was used to obtain DNA fingerprints from different species of the genus *Onchocerca*. Each of the 20 primers tested allowed a clear distinction between the different species of the genus on an agarose gel. The technique offers the potential to construct species or strain specific probes and oligonucleotides PCR primers from the species specific fragments. A combination of these primers or others could be useful as population markers.

Introduction

There are marked geographical variations in the spectrum of disease associated with onchocerciasis. Clinical manifestations ranging from dermal lesions to ocular manifestations including blindness can occur. In West Africa, the incidence of blindness is much higher in savannah areas than in forest areas (Anderson et al., 1974; Prost et al., 1980). In forest areas, despite high prevalence rates and large numbers of microfilariae in the skin, the disease is generally less severe than in savanna areas. An exception is the pattern of disease in Sierra-Leone, where blinding onchocerciasis occurs in forest regions (McMahon et al., 1986). Most onchocerciasis vectors are known to be zoophilic, and can thus be infected with animal *Onchocerca* species whose third stage larvae (L3) are morphologically indistinguishable from each other and from *O. volvulus*. These non-*volvulus Onchocerca* larvae affect the accurate measurement of the transmission of human onchocerciasis and thus, there is a real need for a method which can distinguish between the *Onchocerca* species as well as between strains of *O. volvulus*. This is especially important in areas where onchocerciasis control programmes are developed and where mass distribution of ivermectin is planned.

Immunological and biochemical studies on *O. volvulus* have done little to clarify the possible existence of distinct strains, although allozyme studies showed evidence of genetic variation (Cianchi et al., 1985; Flockhart et al., 1986). However, recently, some DNA probes specific for *O. volvulus* have been described which belong to a tandemly repeated DNA sequence family (Meredith et al., 1989; Harnett et al., 1989). A polymerase chain reaction (PCR), based on the 150 bp repetitive DNA, has now been developed for the detection of all stages of *O. volvulus* (Meredith et al., 1991). The variability of the 150 bp family has been exploited to develop species and strain specific DNA probes and has shown that at least two genetic forms of *O. volvulus* do exist in West Africa (Erttmann et al., 1987; Erttmann et al., 1990). The high level of homology within this repeat family means that the species specificity of these probes is largely dependant on the hybridization and washing stringencies (Zimmermann et al., 1992).

A technique generating genomic fingerprints by Random Amplified Polymorphic DNA (RAPD) based on the amplification by the Polymerase Chain Reaction (PCR) of random DNA fragments has recently been developed. In RAPD mapping, decamer oligonucleotide primers of arbitrary sequence but with a GC content of 50 % or higher are used to amplify fragments of genomic DNA (Williams et al., 1990; Welsh and McClelland, 1990). RAPD has been successfully applied to the analysis of genomic DNA variation of several organisms (Hadrys et al., 1992; Klein-Lankhorst et al., 1991; Crowhurst et al., 1991; Mazurier et al., 1992). Species and strains can be distinguished by comparing polymorphisms in genomic fingerprints (Welsh et al., 1991). The particular advantages of RAPD technology is that small amounts (nanograms) of genomic DNA are needed and, unlike PCR, no prior knowledge of the genome subjected to analysis is required.

We describe here the adaptation of the RAPD assay to the genus *Onchocerca*, using a set of 20 oligonucleotide decamer primers. By comparing the fingerprints generated by the RAPD method, *O. volvulus, O. gutturosa, O. lienalis, O. gibsoni* and *O. ochengi* can be identified and distinguished from each other by the specific polymorphic patterns.

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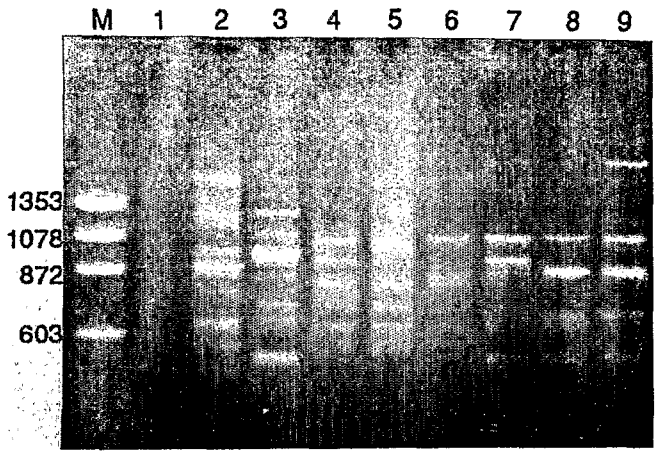

Fig. 1 Single primer PCR on genomic DNA from different species. Primer OPB-4 was used to amplify genomic DNA from human (lane 2), *Dirofilaria immitis* (lane 3), *O. gutturosa* (lane 4), *O. lienalis* (lane 5), *O. gibsoni* (lane 6), *O. ochengi* (lane 7) and two different *O. volvulus isolates* from Cameroon (lane 8) and from Sierra-Leone (lane 9). M: Molecular Weight Marker, ØX-174 Hae III digest. Lane 1: negative control.

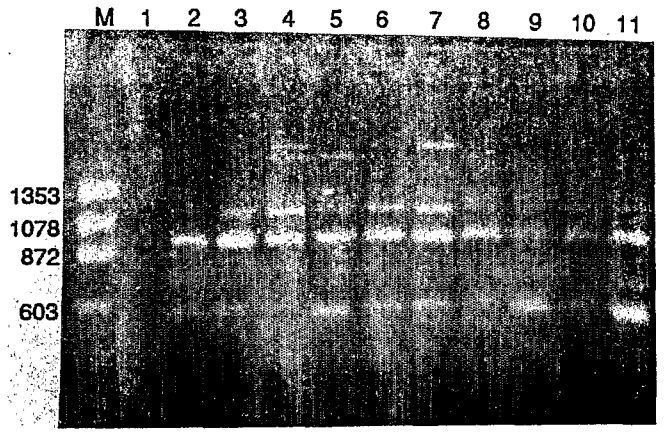

Fig. 2 Single primer PCR on *O. volvulus* genomic DNA from different origins. Genomic DNA from *O. volvulus* (two different worms from the same nodule) coming from Cameroon (4 foci: Touboro, Poli, Bafia and Sa'a) and from Sierra-Leone (1 focus) were amplified with primer OPB-4.
M: Molecular Weight Marker, ØX-174 Hae III digest.
Lane 1: negative control
Lane 2: worm 1 from Touboro
Lane 3: worm 2 from Touboro
Lane 4: worm 1 from Poli
Lane 5: worm 2 from Poli
Lane 6: worm 1 from Bafia
Lane 7: worm 2 from Bafia
Lane 8: worm 1 from Sa'a
Lane 9: worm 2 from Sa'a
Lane 10: worm 1 from Sierra Leone
Lane 11: worm 2 from Sierra Leone

Materials and methods

Nodules containing *O. volvulus* were excised and either conserved directly in absolute alcohol or collagenase-digested (Schultz-Key et al., 1977) and the worms preserved in absolute alcohol. The nodules were from savannah areas (Touboro and Poli, Cameroon), degraded forest areas (Bafia and Sa'a, Cameroon) and the isolated worms from forest regions of West Africa (Lunsar and Njala, Sierra-Leone). *Dirofilaria immitis*, *O. gutturosa*, *O. lienalis*, *O. gibsoni*, and *O. ochengi* DNA were obtained from pooled worms as described previously (Meredith et al., 1989). The *O. volvulus* DNA was extracted from pooled worms as described by Meredith et al. (1989). PCR reactions were performed in 25 µl of 10 mM Tris-HCl (pH 9,0), 50 mM KCl, 0,1 % Triton X-100, 1,5 mM $MgCl_2$, 0,2 mM of each dNTP, 20 picomoles of decamer primer(s) as given in Table **1** (Kit B, RAPD™ primer kits, Operon Technologies Inc.), 25 ng template DNA and 1 unit Taq polymerase (Promega). Amplification was carried out in a thermocycler (Techne PHC-3) programmed for 45 cycles of 1 minute at 92 °C, 1 minute at 36 °C and 2 minutes at 72 °C. Amplification products were resolved electrophoretically on 2 % agarose gel, revealed by ethidium bromide and photographed under U. V. light (Sambrook et al., 1989).

Table 1 Nucleotide sequence of primers OPB-01 to OPB-20 (Kit B, Operon technologies Inc.).

Primer	Nucleotide sequence
OPB-01	5' GTTTCGCTCC 3'
OPB-03	5' CATCCCCCTG 3'
OPB-04	5' GGACTGGAGT 3'
OPB-05	5' TGCGCCCTTC 3'
OPB-06	5' TGCTCTGCCC 3'
OPB-07	5' GGTGACGCAG 3'
OPB-08	5' GTCCACACGG 3'
OPB-09	5' TGGGGGACTC 3'
OPB-10	5' CTGCTGGGAC 3'
OPB-11	5' GTAGACCCGT 3'
OPB-12	5' CCTTGACGCA 3'
OPB-13	5' TTCCCCCGCT 3'
OPB-14	5' TCCGCTCTGG 3'
OPB-15	5' GGAGGGTGTT 3'
OPB-16	5' TTTGCCCGGA 3'
OPB-17	5' AGGGAACGAG 3'
OPB-18	5' CCACAGCAGT 3'
OPB-19	5' ACCCCCGAAG 3'
OPB-20	5' GGACCCTTAC 3'

Abbreviations

Deoxyribonucleic acid (DNA); random amplified polymorphic DNA (RAPD); base pairs (bp): polymerase chain reaction (PRC); sodium lauryl sulfate (SDS); operon primer kit B (OPB).

Results

First, we investigated the potential of RAPD in differentiating at the species level. Genomic DNA from different filarial species was amplified by use of a single decamer primer; 20 primers were tested in these experiments and in each case, at least one specific fragment was obtained for each *Onchocerca* DNA used. Fig. **1** shows the amplifications with primer OPB-4 on DNA from Human, *D. immitis*, *O. gutturosa*, *O. lienalis*, *O. gibsoni*, *O. ochengi* and two of *O. volvulus* isolates from Cameroon and Sierra-Leone. Depending on the primer used, several bands can be seen, ranging from 1.5 Kb to 150 bp. Some of the bands are common between the different species and some are unique to one species. Preliminary investigations were also carried out to determine whether the RAPD patterns generated by these primers (OPB-1 to OPB-20) could be useful in strain differentiation within *O. volvulus*.

DNA from different *O. volvulus* worms (4 isolates from Cameroon and 1 from Sierra-Leone) were amplified with different primers: Fig. 2 shows these amplifications with primer OPB-4; this figure is representative of all the amplification profiles obtained with the 19 other primers. The patterns observed for the different worms are very similar with some differences represented only by low intensity bands. To increase the chance of detecting intraspecific variation, pairs of oligonucleotides were tested. As an example, amplifications of *O. volvulus* genomic DNA with two primers (OPB-7 and 8) are presented in Fig. 3: the combination of two primers (Lane 2) result in the appearance of new amplified DNA fragments compared to amplification with single primer (Lane 1 and 3).

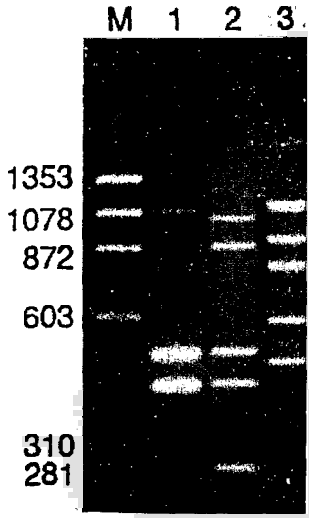

Fig. 3 Comparison of patterns generated with single and mixed primers (*O. volvu-lus* from Sierra-Leone).
M: Molecular Weight Marker, ØX-174 Hae III digest.
Lane 1: genomic DNA amplified with primer OPB-7.
Lane 2: genomic DNA amplified with primer OPB-7 and OPB-8.
Lane 3: genomic DNA amplified with primer OPB-8.

Discussion

RAPD is useful in differentiating at genus, species, and intraspecific level (Hadrys et al., 1992). Specific RAPD markers have been identified at genus level and species level in irisis (Arnold et al., 1991) and tomato (Klein-Lankhorst et al., 1991), strain level in mouse (Welsh et al., 1991), "cultivar" level in broccoli and cauliflower (Hu and Quiros, 1991) and clone level in fungi (Smith et al., 1992). Divergence of even a fraction of one percent between two genomes often results in a different fingerprinting and thus allow strains of almost any organism to be distinguished (Welsh et al., 1991).

In this study, fragments of genomic DNA from various species of the genus *Onchocerca* were obtained by amplification with short primers of arbitrary nucleotide sequence. The polymorphic patterns allowed a clear distinction between the different species by RAPD with numerous different primers. When standardized reaction conditions were used, these results were highly reproducible. Clear differences on a simple agarose gel can be observed, unlike the conventional PCR assay described for *O. volvulus* (Meredith et al., 1991) that necessitate Southern blot analysis and hybridization. One important problem in Onchocerciasis control strategies and surveillance is the identification of *Onchocerca* species infecting the *Simulium* vector. The RAPD technique is not suitable for identification of the parasites in the fly since polymorphic pattern of the vector DNA will be superposed with those of the parasite, making interpretation of results difficult. However it shows great potential for the generation of species specific probes from the fragments generated, and species specific PCRs could be developed from the unique fragments for direct identification of *Onchocerca* species in vectors.

The primers used in this study do not show clear distinctions between the different isolates of *O. volvulus* tested. However, considering the number of RAPD primers available it is more than likely that clear differentiation between isolates can be obtained with other primers. Thus, a combination of these or other random primers could prove to be useful population markers. Further investigation of large number of isolates of *O. volvulus* is needed to evaluate the potential of RAPD in studies on population genetics. This technique may allow the selection of species or strain specific sequences as potential probes more easily than the classical approach. Such probes could improve the direct identification of *Onchocerca* infected vector (by hybridization or PCR) and the specificity would not be dependant on the stringency conditions.

It is important to be able to identify the origin or form of the parasite (L3 of different species of the genus *Onchocerca* or different strains of *O. volvulus*) in order to plan appropriate onchocerciasis control strategies. The results obtained on the correlation between classification of *O. volvulus* by DNA probes and epidemiologic patterns of onchocercal blindness (Zimmerman et al., 1992), support the hypothesis of strain association with pathogenicity.

RAPD analysis is interesting because very small amounts of genomic DNA are subjected to PCR and the amplification products are resolved on agarose gel. Consequently, for field application, there is no need for utilisation of radioactivity. Polymorphisms generated by this assay are useful as genetic markers when strictly standardized reaction conditions are used (Hadrys et al., 1992), which guarantee reproducible amplification products.

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References

Anderson, J., H. Fuglsang, P. J. S. Hamilton, T. F. Marshall: Studies on onchocerciasis in the United Cameroon Republic, II. Comparison of onchocerciasis in rain forest an Sudan savanna. Trans. R. Soc. Trop. Med. Hyg. 68 (1974) 209–222

Arnold, M. L., C. M. Buckner, J. J. Robinson: Pollen-mediated introgression and hybrid speciation in Louisiana irises. Proc. Natl. Acad. Sci. USA 88 (1991) 1398–1402

Cianchi, R., M. Karam, M. C. Henry, F. Villani, S. Kumelien, L. Bullini: Preliminary data on the genetic differenciation of *Onchocerca volvulus* in Africa (Nematoda: Filarioidea). Acta Tropica 42 (1985) 341–351

Crowhurst, R. N., B. T. Hawthorne, E. H. Rikkerink, M. D. Templeton: Differentiation of *Fusarium solani* f. sp. *cucurbitae* races 1 and 2 by random amplification of polymorphic DNA. Current Genet. 20 (1991) 391–396

Erttmann, K. D., T. R. Unnasch, B. M. Greene, E. J. Albiez, J. Boateng, A. M. Denke, J. J. Ferraroni, M. Karam, Schulz-Key, P. N. Williams: A DNA sequence specific for forest form *Onchocerca volvulus*. Nature 327 (1987) 415–417

Erttmann, K. D., S. E. O. Meredith, B. M. Greene, T. R. Unnasch: Isolation and characterisation of form specific DNA sequences of *O. volvulus*. Acta Leidensia 1–2 (1990) 253–260

Flockhart, H. A., R. E. Cibulskis, M. Karam, E. J. Albiez: Onchocerca volvulus: enzyme polymorphism in relation to the differenciation of forest and savannah strains of this parasite. Trans. R. Soc. Trop. Med. Hyg. 80 (1986) 285–292

Hadrys, H., M. Balick, B. Schierwater: Applications of random amplified polymorphic DNA (RAPD) in molecular ecology. Mol. Ecol. 1 (1992) 55–63

Harnett, W., A. E. Chambers, A. Renz, R. M. E. Parkhouse: An oligonucleotide probe specific for *Onchocerca volvulus*. Mol. Biochem. Parsitol. 35 (1989) 119–126

Hu, J., C. F. Quiros: Identification of broccoli and cauliflowers cultivars with RAPD markers. Plant Cell Report 10 (1991) 505–511

Klein-Lankhorst, R. M., A. Vermunt, R. Weide, T. Liharska, P. Zabel: Isolation of molecular markers for tomato (*L. esculentum*) using random amplified polymorphic DNA (RAPD). Theor. Appl. Genet. 83 (1991) 108–114

Mazurier, S., A. van de Giessen, K. Heuvelman, K. Wernars: RAPD analysis of *Campylobacter* isolates: DNA fingerprinting without the need to purify DNA. Letters in Appl. Microbiol. 14 (1992) 260–262

McMahon, J. E., J. B. Davies, M. D. White, J. M. Goddard, P. A. Beech-Garwood, B. R. Kirkwood: Onchocerciasis in Sierra-Leone. I. Studies on the prevalence and transmission at Gbaiima village. Trans. R. Soc. Trop. Med. Hyg. 80 (1986) 802–809

Meredith, S. E. O., T. R. Unnasch, M. Karam, W. F. Piessens, D. F. Wirth: Cloning and characterisation of an *Onchocerca volvulus* specific DNA sequence. Mol. Biochem. Parasitol. 36 (1989) 1–10

Meredith, S. E. O., G. Lando, A. A. Gbakima, P. A. Zimmermann, T. R. Unnasch: Onchocerca volvulus: application of polymerase chain reaction to identification and strain differentiation of the parasite. Exp. Parasitol. 73 (1991) 335–344

Prost, A., A. Rougemont, M. S. Omar: Caractères épidémiologiques, cliniques et biologiques des onchocercoses de savane et de forêt en Afrique Occidentale. Ann. Parasitol. 55 (1980) 347–355

Sambrook, J., F. F. Fritsch, T. Maniatis: Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989). Second Edition

Schultz-Key, H., F. J. Albiez, D. W. Büttner: Isolation of living adult *Onchocerca volvulus* from nodules. Tropenmed. Parasitol. 28 (1977) 428–430

Smith, M.L., J. N. Bruhn, J. B. Anderson: The fungus *Armillaria bulbosa* is among the largest and oldest living organisms. Nature 356 (1992) 428–431

Welsh, J., M. McClelland: Fingerprinting genomes using PCR with arbitrary primers. Nucl. Acid Res. 18 (1990) 7213–7218

Welsh, J., C. Petersen, M. McClelland: Polymorphisms generated by arbitrarily primed PCR in the mouse: application to strain identification and genetic mapping. Nucl. Acid Res. 19 (1991) 303–306

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski, S. V. Tingey: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucl. Acid Res. 18 (1990) 6531–6535

Zimmerman, P. A., K. Y. Dadzie, G. de Sole, J. Remme, E. Soumbey Alley, T. R. Unnasch: Onchocerca volvulus DNA probe classification correlates with epidemiologic patterns of blindness. J. Infect. Dis. 165 (1992) 964–968

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