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ISOLATION OF NEW MARKERS TO DETECT GENETIC VARIATION IN *ONCHOCERCA VOLVULUS*

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KEYWORDS : *Onchocerca volvulus*. genetic variability. RAPD. microsatellite. PCR.

SUMMARY

Newly developed techniques (RAPD : Random Amplified Polymorphic DNA and microsatellite DNA sequences) were used to study genetic

variation in *Onchocerca volvulus*. RAPD technique, derived from the Polymerase Chain Reaction (PCR), allow clear distinction between the different species of the genus *Onchocerca*.

Microsatellite DNA has been shown to be useful as polymorphic markers for populations and even for individuals. Short repeated sequences (CA repeats) have been isolated, and a PCR assay using such microsatellite DNA sequences to generate polymorphisms is currently being experimented.

In West Africa, particularly in the OCP area (Onchocerciasis Control Programme), at least two strains of the parasite are known to exist. The so-called "savannah" strain associated with high blindness level, and the "forest" strain associated with a milder form of onchocerciasis which causes blindness in less than 1 % of the population (Prost *et al.*, 1980). However, Sierra Leone is exceptional in that high levels of blindness are found in forest areas (McMahon *et al.*, 1986).

Various *Onchocerca* specific DNA probes have been developed (Meredith *et al.*, 1989) and also strain specific probes (Erttmann *et al.*, 1987 and 1990). These DNA probes sequences are based on the 150 bp repeat family. Recently, studies conducted in West Africa have shown that DNA probe classification correlates with the epidemiology of blindness (Zimmerman *et al.*, 1992).

In Cameroon, the situation is more complex, notably the pathology of blindness is distributed without apparent relation to bioclimatic zones (Duke, 1981 ; Boussinesq *et al.*, 1993). To date, no studies similar to those carried out in West Africa have been done in Cameroon.

We are investigating other molecular markers that may be useful for studies on genetic variation in *O. volvulus*, namely RAPD and microsatellite sequences.

Polymorphism in genomic fingerprints generated by Random Amplified Polymorphic DNA (RAPD) is a recently developed assay that is based on the amplification by the Polymerase Chain Reaction (PCR) of random DNA fragments. 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 (Williams *et al.*, 1990 ; Hadrys *et al.*, 1992 ; Klein-Lankhorst *et al.*, 1991 ; Crowhurst *et al.*, 1991 ; Mazurier *et al.*, 1992). 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 sequence knowledge is needed.

Microsatellite DNA sequences are short (from 2 to 5 bp), tandemly repeated sequences that have been shown to be useful as polymorphic markers for populations and even for individuals. They have been reported from a large panel of eukaryotic species such as human, mouse, cattle, whales and insects (Tautz, 1989 ; Love *et al.*, 1990 ; Swaiger *et al.*, 1992 ; Weber and May, 1989 ; Hughes and Queller, 1993). These dinucleotide repeats are referred to as microsatellites (Litt and Luty, 1989). Most of these regions are less than 200 bp in length and PCR is used to detect length polymorphisms.

We describe here the use of the RAPD assay for the detection of variation in the genus *Onchocerca*, using a set of 20

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oligonucleotide decamer primers and the adaptation of microsatellite DNA sequences to study genetic variability in *O. volvulus* populations.

METHODS

RAPD

Nodules containing *O. volvulus* were excised and collagenase-digested (Schulz-Key *et al.*, 1977) and the worms preserved in absolute alcohol. The nodules were from savannah and degraded forest areas of Cameroon (Toubo, Poli and Bafia, Sa'a) and from forest regions of Sierra Leone (Lunsar and Njala).

O. gutturosa, *O. lienalis*, *O. gibsoni* and *O. ochengi* DNA was provided by Dr. Meredith. *O. volvulus* DNA was extracted from worms as described by Meredith *et al.* (1991). PCR reactions were performed in 25 µl of 10mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 % Triton X-100, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 20 picomoles of decamer primer(s) (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 1.4 % agarose gel.

MICROSATELLITE DNA SEQUENCES

Nodules containing *O. volvulus* worms from 14 patients originating from 4 different foci in Cameroon were excised and collagenase digested in the field. Total genomic DNA from the worms was digested to completion with the restriction enzymes *Hae*III, *Alu*I, *Taq*I, *Hinf*I and *Sau*3A and shotgun cloned under standard conditions into a M13BM20 vector containing an *Eco*RV site (Hanahan, 1983). Poly(CA)_n/(GT)_n and poly(GA)_n/(CT)_n probes for detection of respectively (CA)_n/(GT)_n and (GA)_n/(CT)_n microsatellites sequences were labelled by random priming in the presence of [α -³²P]dCTP (Feinberg and Vogelstein, 1983). The genomic M13 library was screened by transferring the plaques onto nylon membranes. Prehybridization and hybridization were performed at 65° C in a solution containing 0.5 % nonfat powdered milk, 5 X SSPE, 1 % SDS, 100 µg/ml denatured salmon sperm DNA, followed by two washes of 15 minutes each in 2 X SSPE, 0.1 % SDS, one of 15 minutes in 0.5 X SSPE, 0.1 % SDS and the stringent wash of 15 minutes in 0.1 X SSPE, 0.1 % SDS at 65° C. Dried filters were then autoradiographed with an intensifying screen on Hyperfilm (Amersham) at -70° C for 2 to 16 hours. Single-stranded template DNA was isolated from positive plaques (Messing, 1983) and sequenced (Sanger, 1977) on an automatic sequencer (Applied Biosystems, Inc.).

PCR primers were chosen from the region immediately flanking the microsatellite sequence. These primers were designed with computer assistance to minimize self annealing. PCR conditions were optimized by combining different Mg²⁺ concentrations, annealing temperatures and number of amplification cycles. PCR products were resolved on non-denaturing acrylamide gels (from 7 to 10 %) and visualised by ethidium bromide (1 mg/ml) or silver staining

(0.2 % silver nitrate for 30 minutes and reduction with 0.75M NaOH ; 0.1M formaldehyde for 10 minutes).

RESULTS AND DISCUSSION

First, we investigated the potential of RAPD in differentiating at the species level. Genomic DNA from different 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 the *Onchocerca* DNA's used. For each sample, some of the bands (ranging from 1.5 Kb and 150 bp) are common for the genus *Onchocerca* and some are unique to one species depending on the primer used. By comparing fingerprints generated by the RAPD method, *Onchocerca volvulus*, *O. gutturosa*, *O. lienalis*, *O. gibsoni* and *O. ochengi* can be identified and distinguished from each other by the specific polymorphic patterns.

Secondly, RAPD profiles of different *O. volvulus* isolates have been compared using the same primer as described earlier. Different patterns are observed between different isolates and even between worms from the same nodule.

Using these primers, we could observe differences between species and also between *O. volvulus* isolates. However when amplification is repeated, we often find the profiles to vary slightly. Furthermore, the presence of DNA contamination can seriously affect the results. Thus, in this case, this technique seems more suitable for isolation of new DNA probes rather than for studies on population genetic.

Microsatellite loci can be easily scored using polymerase chain reaction (PCR) followed by electrophoresis to separate alleles which differ in length as a result of differences in the number of repeat units. The microsatellite sequences isolated from *O. volvulus* have been used to make primers (microsatellite flanking regions) to identify different strains of the parasite. These particular sequences consist of CA repeats. Initial results using *O. volvulus* microsatellite primers shows only slight differences between isolates. This lack of resolution observed between bands representing alleles (in some cases separated only by 2 bp) can be explained by the use of non denaturing acrylamide gel to visualize amplification products. In order to allow better resolution of DNA fragments, this technique can be improved by using denaturing sequence acrylamide gels.

In conclusion, we believe that these new tools developed (particularly microsatellites) will be useful for investigating the genetic variation in *O. volvulus*.

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CUTICLIN GENES OF NEMATODES

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KEYWORDS : parasitic nematodes, cuticle, cuticlin, dityrosine, cross-linking.

SUMMARY

Two genes coding for cuticlin components of *Caenorhabditis elegans* have been cloned and their structure is described. Recombinant proteins have been produced in *E. coli* and antibodies raised against them. Nucleic acid and specific antibodies are being used to isolate the homologues from the parasitic species *Ascaris lumbricoides* and *Brugia pahangi*.

The nematode cuticle protects the animal, serves as an exoskeleton and provides the surface over which interactions with the external environment occur. In the case of parasitic nematodes the external environment is the host and, as such, antigens expressed on/in the cuticle are within reach of both the humoral and cellular components of the immune system. The cuticle is a layered structure, the components of which are classified according to their solubility : lipids, some proteins and other readily soluble, non-structural components are mostly localized on the surface but are also distributed to a lesser extent throughout the lower layers of the cuticle ; the collagens make up the structural bulk of the cuticle, are coded for by a large and relatively well-characterized gene family, are not exposed on the cuticle surface and can be solubilized with SDS and mercaptoethanol ; and finally there is a highly cross-linked, insoluble and complex mixture of proteins present throughout the cuticle and known as the cuticlins. Up until now it has been virtually impossible to determine the roles and importance of the cuticlins because their insolubility does not allow either molecular or biochemical analysis of individual proteins.

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