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A FIELD STUDY USING THE POLYMERASE CHAIN REACTION (PCR) TO SCREEN FOR BRUGIA MICROFILARIAE IN HUMAN AND ANIMAL BLOOD

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ABSTRACT

Blood samples from 43 humans and 14 cats positive with Brugia microfilariae were analyzed in a field study in Tanjung Pinang, Indonesia. The study used the polymerase chain reaction (PCR) to compare the sensitivity of radioactive and biotinylated species-specific oligonuleotide probes. The cloning characterization of the Hha I repeat DNA family found in filarial parasites of the genus Brugia, and the development of species-specific probes for B.malayi and B.pahangi based on these repeats has been described elsewhere (PNAS USA 83: 797-801); Mol.Biochem. Parasitol. 28: 163-170). The use of radioisotopes for labelling DNA probes is both expensive and inconvenient. To replace these probes, biotinylated DNA probes have been designed for non- radioactive detection of B.malayi and B.pahangi. These oligonucleotide probes have long tails of biotinylated uridine residues added to their 5' end. As little as 100 pg of Brugia DNA can be detected on dot blot with these probes. Detection of the probes is based on an avidin-alkaline phosphatase colorimetric assay. In order to distinguish between infected from uninfected individuals, it is necessary to detect the amount of DNA in one microfilaria (about 60 pg). The polymerase chain reaction (PCR) is a procedure in which a small amount of DNA can be amplified up to 1 million-fold. A part of each sample in this study was PCR amplified and compared with the unamplified portion using both the radioactive and biotinylated DNA probe.

The PCR amplified samples were accurately identified by both the radioactive and biotinylated B.malayi and B.pahangi probes. Even samples with as few as two microfilariae per 100ul of blood were easily detected. The samples that were not PCR amplified were accurately identified after only long exposures (greater than one week) to the radioactive probes. The biotinylated probes, were not sensitive enough for accurate identification of the non-PCR amplified samples. The polymerase chain reaction is, therefore, a promising new tool for enhancing the sensitivity of parasite detection assays based on DNA probes. This will be especially important in designing assay based on non-radioactive DNA probes.

INTRODUCTION

Filarial parasites are of considerable importance in the developing nations of the world, infecting millions of people[1]. All species are transmitted by an arthropod vector, most of which deposit larvae on the ma-

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mmalian host during bloodmeals. The disease in man, filariasis, results in a lymphatic infection that can lead to the end-stage deformity, elephantiasis. Most lymphatic filariasis cases in man are caused by *Brugia malayi* and *Wuchereria bancrofti.* Experimental infections of man by *B.pahangi* have also been reported[2]. Areas endemic for *B.malayi* and *B.pahangi* overlap, and both species can be carried by some of the same mosquito vectors[3].

Currently, there is no rapid assay for distinguishing closely related species of filarial worms. There are several diagnostic methods, ranging from simple blood counting to complex serological tests. All methods have varying degrees of sensivity. The ideal field and/or laboratory assay for detection of filariae would be easy to use, fast and inexpensive. The technique should involve a minimum of sophisticated equipment and utilize stable reagents. In addition, the assay should give uncomplicated and reproducible results.

A species-specific diagnostic assay using DNA repeat sequences cloned from *Brugia* has been developed. Cloned repeated DNA has been used as a probe for the detection of other eukaryotic parasites[4,5,6,7]. The cloning and characterization of the Hha I 322 base pair repeat family found in *Brugia* parasites and their use as probes has been described elsewhere[8,9]. Previously, the assay used radioisotopes for labeling the DNA probes. This is both expensive and inconvenient for use in field studies. To replace these probes, biotinylated and horseradish peroxidase labeled DNA oligonucleotide probes have been constructed for non-radioactive detection of *Brugia* species.

For the purposes of epidemiological studies in endemic regions, it is necessary to identify infected and uninfected individuals. Therefore, an assay for widespread use must be able to detect approximately 100 pg of DNA, the amount found in one microfilaria. Use of the polymerase chain reaction (PCR) allows up to a one million fold amplification of small DNA quantities. Such an amplification could greatly enhance the signal in detecting low microfilariae titres.

In this report, human and cat blood samples from Tanjung Pinang, Indonesia, were analyzed using PCR to compare the sensitivity of radioactive and non-radioactive oligonucleotide probes. Two non-radioactive detection systems were tested: an avidin-alkaline phosphatase colorimetric assay to detect the biotinylated probes, and a cyclic diacylhydrazide-peroxidase chemiluminescence assay to detect the horseradish peroxidase labeled DNA probes.

MATERIALS AND METHODS

Human and cat blood samples were collected in a field study in Tanjung Pinang, Indonesia. The oligonucleotide DNA probes for detecting *B.malayi* and *B.pahangi* and the primers used for PCR amplification were synthesized using the phosphoramidite method[10]. The probes were designed from the sequence of the *Hha* I repeat DNA family that is present in all *Brugia* species. The 322 base pair repeat sequences in *B.malayi* and *B.pahangi* are 90 % homologous. Within the repeat sequence, there exist a short 66 base pair region of divergence between the species where the sequence homology is only 72 %[8]. Based on this repeat sequence data, oligonucleotide probes were designed that maximized the amount of sequence difference between the species. The sequence homology of the 45 nucleotides long probes to the probe of the opposite species was only 57 % . A 61 nucleotide tail of biotinylated uridine residues alternated with thymidine

residues was added to the 5' end of each of the two probes. Two primers, an 18- and a 21-nucleotide long oligonucleotide representing the beginning and end of the repeat sequence, were used for PCR amplification. Microfilarial DNA standards were prepared from parasite material supplied by John Mc-Call (TRS Laboratories, Athens, GA).

Digestion of microfilariae and DNA isolation

Blood samples were collected from humans and cats in villages of Tanjung Pinang, Indonesia, by venepuncture. These 500 µl samples were stored with 160 µl of 0.5 M EDTA in PBS to prevent DNA degradation and blood clotting. One 100 µl aliquot was removed from each of the 44 human and 13 cat samples after vortexing to disperse any blood clots. These 100 µl samples were transfered to sterile 1.5 ml microcentrifuge tubes. The samples were digested in a cocktail containing 0.5 % sodium dodecyl sulfate (Sigma Chemical Company, St. Louis, MO), 50 mM betamercaptoethanol (Sigma), 100 µg/µl proteinase K (Bethesda Research Labora-

tories, Gaithersburg, MD) and TE (10mM TRIS, 1 mM EDTA) in a total volume of 500 µl for 4 hours at 65^0C. After phenol extraction (twice with TRIS-saturated phenol and twice with chlorofom-saturated phenol[11], the aqueous layer volume was evaporated to 100 µl and dialyzed against TE for 6 hours on 0.025 um pore filters (Millipore Corporation, Bedford, MA).

PCR amplification

Five microliters of each sample were used for PCR amplification using the enzyme *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT). The samples were amplified in a DNA Thermal Cycler (Perkin-Elmer Cetus) using a standard 25-cycle program. The PCR cycle consists of a DNA melt at 94^0C for 1 minute to separate double strands, a template-primer annealing reaction at 37^0C for 1 minute, and the polymerization reaction at 72^0C for 3 minutes[12-15]. To determine if amplification had occured, 10 µl of each PCR amplified sample was electrophoresed on a 1.5 % agarose gel.

Figure 1. Map of PCR and non-PCR (unamplified) samples on the DNA dot blots

	A	B	C	D	E	F	G	H	I	J	K	L
1	M5	M10	M25	M50	P5	P10	P25	P50	C49	C82	C87	C90
2	C122	C127	C104	C107	C108	C109	C111	C112	C114	28	33	37
3	39	42	134	139	150	153	156	160	175	186	279	281
4	298	391	393	395	399	402	408	410	411	412	421	423
5	427	428	429	432	433	434	435	437	450	452	309	310
6	318B	367	383	384	445							

"M" samples (A1, B1, C1, D1) represent DNA isolated from *B.malayi* purified control samples: 5, 10, 25 and 50 microfilariae (mf). "P" samples (E1, F1, G1 and H1) represent *B.pahangi* DNA isolated from control purified samples in the same concentrations as listed above. Control purified mf were obtained from John McCall from TRS Laboratories (Atlanta, GA). "C" samples are cat samples. The samples are grouped and numbered by villages. Spots that are circled represent samples with less than 15 mf per 20 µl blood (see Table 1).

Detection of Brugia DNA with radioactive oligonucleotide probes

Both PCR amplified and non-PCR (unamplified) samples were prepared by the method of Kafatos, *et al*[16]. The DNA samples were denatured in 0.3 N sodium hydroxide for 10 minutes at 25^0C (room temperature), neutralized and precipitated in 2M ammonium acetate on ice and spotted onto 0.45 um pore nylon membranes (Nytran, Schleicher and Schuell, Keene, NH) in a 96-well dot blot apparatus (Bio-Rad, Richmond, CA). Two microliters of each PCR reaction and 20 µl of each non-PCR (unamplified) sample was used for dot blot analysis. Following filtration of the sample, each well was flushed with 200 µl 2M ammonium acetate. The filters were baked at 60^0C under vacuum for 2 hours.

For direct comparison with radioisotope detection methods, the biotinylated *B.malayi* and *B.pahangi* probes were 5' endlabeled with T4 polynucleotide kinase and [gamma-32p]ATP[17] to an approximate specific activity of $1x10^9$ counts min^{-1} mg^{-1} of oligonucleotide DNA. The blots were pre-hybridized for 2 hours in 50% deionized formamide, 5X SSC, 5X Denhardt's, 25mM sodium phosphate pH 6.5 and 500 µg/ml denatured salmon sperm DNA[11]. Overnight hybridization in 45% deionized formamide, 5X SSC, 1X Denhardt's, 20 mM sodium phosphate pH 6.5, and 200 µg/ml denatured salmon sperm DNA[11] occured at 37^0C with the addition of $2.6x10^6$ cpm per ml hybridization solution of either the *B.malayi* or *B.pahangi* DNA probes. Following hybridization, the filters were washed in 2X SSC twice at room temperature for 30 minutes, once at 60^0C for 30 minutes, once at 63^0C for 30 minutes, once at 65^0C for 1 hour and once at 68^0C for 45 minutes. The blots were covered with saran wrap and expose to autoradiography film for 3 hours (Eastman-Kodak, Rochester, NY).

Detection of Brugia DNA with biotinylated probes

PCR amplified DNA was also detected in a colorimetric assay. These blots were prepared as above except that the hybridization occurred at 39^0C using 25 ng of biotinylated oligonucleotide probe per ml hybridization solution. The filters were washed post-hybridization in 3X SSC once for 30 minutes each at 25^0C, 39^0C and 65^0C. The presence of biotin on the oligonucleotide was detected using an avidin-alkaline phosphatase detection system (Vectastain ABC detection system, Vector Laboratories, Burlingame, CA)[18,19].

Detection of Brugia DNA using Chemiluminescence

The same biotinylated DNA probes were also labeled by the enhanced chemiluminescence method (ECL gene detection system, Amersham Corporation, Arlington Heights, IL). In the labeling procedure, molecules of horseradish peroxidase are covalently linked into the DNA by glutaraldehyde treatment The enzyme catalyzes the oxidation of luminol in the presence of hydrogen peroxide. The result of this reaction is a light emission which can decay silver grains on autoradiography film. Two new dot blots of the PCR samples were prepared as outlined above except that nitrocellulose membranes (Schleicher and Schuell, Keene, NH) were used instead of nylon. Labeling of the DNA probes, pre- hybridization, hybridization, post-hybridization washes and detection were carried out using Amersham ECL reagents and protocols[20,21,22].

RESULTS

PCR amplification allowed visualization of DNA from all samples, including those with very low parasite titres. The number of microfilariae in each sample was previously determined by microscope counting of blood smears. *Hha* I repeat DNA was successfully amplified from each sample using PCR, as shown by gel electrophoresis (data not shown). Each human sample in the study had at least two microfilariae per 20 μl blood. Microscope data for the cat samples is not yet available.

The species specificity of the probes was demonstrated by hybridization to *Brugia* DNA that had been isolated from purified microfilariae and amplified. The *B.pahangi* probe hybridized strongly to *B.pahangi* amplified genomic DNA but not to *B.malayi* DNA. The *B.malayi* probe hybridized strongly to *B.malayi* amplified genomic DNA and showed a low level of cross hybridization to *B.pahangi* in the control samples. Perhaps the two samples that hybridized to the *B.pahangi* probes are cats with mixed infections, consisting of a high *B.malayi* microfilariae titre and a low *B.pahangi* titre. Dot blot hybridizations of non-PCR samples with the radioactive-labeled oligonucleotide probe gave poor results. Detection was evident only in cases of extremely high parasites burdens (100-200 microfilariae per 20 μl blood). Detection using the biotin-avidin-alkaline phosphatase colorimetric assay on the non-PCR dot blots yielded similar results (data not shown).

PCR blots hybridized with the radioactive probes yielded species specific and sensitive results. This labeling method allows detection of very low parasite titres. The biotinylated probe in the colorimetric assay allows species specific detection but is less sensitive than either radioactive or chemiluminescent detection (see figure 1, and Table 1). In the samples with titres of 15 microfilariae or less, the hybridization sensitivity radioactive and chemiluminescent detection are equivalent.

DISCUSSION

The usefulness of repeat DNA families as probes for distinguishing between two closely related species has been demonstrated in earlier work[4,7,8,9]. In this report, we have compared two non-radioactive methods for labeling oligonucleotide probes with the standard radioactive labeling method. Because of the large amount of sequence homology between *B.malayi* and *B.pahangi*, cloned repeats of one species hybridized strongly to DNA of the other species[6]. Analysis of repeats cloned from various *Brugia* isolates has enabled the identification of a region of sequence divergence between the *Hha* I repeats of *B.malayi* and *B.pahangi*. It is the use of this area of sequence divergence that gives the oligonucleotide probes property of species specificity.

It is critical for epidemiological analyses to be able to detect 100 pg of DNA, the estimated total amount of DNA in one microfilaria. Humans and animals with low microfilarial titres are often undetected in standard diagnostic procedures. PCR amplification has enabled detection of samples with very low microfilarial titres that could not be detected in unamplified samples hybridized with the ^{32}P-labeled probes.

PCR is not strictly quantitative in its amplification. Several samples with low microfilarial titres amplified greatly while other samples with higher microfilarial titres yielded only modest amounts of DNA. Low level amplifications of samples with high

titres may be due to the higher amount of DNA put into reaction and/or trace contaminants in the samples. Primer binding sites may be blocked in concentrated DNA samples, which could cause lowered PCR efficiency. New methods for making PCR amplification more quantitatively are being developed and will be applied in future field studies.

Radioisotopes are the most commonly used molecules for labeling DNA. This method gives highly sensitive, consistent, and species specific results. However, radioisotope instability, expense, and dangers make this method impractical for use in a field study. In addition, results often take days to obtain. Biotinylation of DNA probes is the most commonly used non-radioactive method and eliminates the problems associated with radioisotope use. However, results from biotin detection tend to be inconsistent and do not give the required sensitivity.

Enhanced chemiluminescence is an improvement over a number of non-radioactive DNA labeling systems, including biotinylation. This method has advantages in sensitivity, speed and consistency. Linking horseradish peroxidase onto DNA probes with glutaraldehyde takes only 15 minutes and does not require DNA purification. The reagents involved are non-hazardous and results can be obtained in less than one hour following hybridization. This labeling method appears to be the most promising for a non- radioactive field assay.

These experiments demonstrate that short synthetic oligonucleotide probes developed from an analysis of sequence divergence between repeat DNA families can be used in distinguishing two closely related species. These probes can be labeled and detected in non-radioactive systems that are as specific and sensitive as traditional radioactive detection methods. Amplification of DNA samples by PCR introduces a way to more accurately identify low microfilarial carriers. The equipment required for PCR amplification is costly, but PCR amplification can be performed manually without a thermal cycler. All of the experiments described utilize purified DNA samples, which requires hours of effort. However, preliminary work has shown that horseradish peroxidase-labeled probes can be used to detect DNA in crude microfilarial lysates in a dot blot hybridization. The method of constructing species specific probes based on highly repeated DNA sequences, the amplification of DNA and a highly sensitive non-radioactive DNA labeling system should prove to be important in the design of DNA assays for the detection of various parasite species.

Table 1. Samples with low microfilarial titres

sample no.	mf count (per 20 μl)
33	6
134	7
139	11
150	5
153	11
160	12
175	4
310	12
367	2
383	5
384	3
395	5
411	11
421	3
423	4
428	13
433	5
434	6
437	2
445	15
452	8

REFERENCES

1. Denham, D.A. and P.B. McGreevy (1977). Brugian filariasis: Epidemiological and experimental studies. Adv.Parasitol. **15**: 243- 309.

2. Edeson, J., T. Wilson, R. Wharton, and A. Laing (1960). Experimental transmission of *Brugia malayi* and *B.pahangi* to man. Trans. R. Soc. Med. Hyg. **54**: 229-234.

3. Palmiri, J., S. Ratiwayanto, S. Masbar, S. Tirtokusumo, J. Rusch, and H. Marwoto (1985). Evidence of possible natural infections of man with *Brugia pahangi* in South Kalimantan (Borneo), Indonesia. Trop. Geogr. Med. **37**: 239-244.

4. Gonzalez, A., E. Prediger, M.E. Huesus, N. Nogeira, and P.M. Lizardi (1984). Minichromosomal repetitive DNA in *Trypanosoma cruzi* Its use in a high-sensitivity parasite detection assay. Proc. Natl. Acad. Sci. USA **81**: 3356-3360.

5. Barker, D.C., and J. Butcher (1983). The use of DNA probes in the identification of leishmaniasis: discrimination between isolates of the *Leishmania mexicana* and *L.braziliensis* complexes. Trans. R. Soc. Trop. Med. Hyg. **77**: 285-297.

6. Barker, R., L. Suebsaeng, W. Rooney, G. Alecrim, H. Dourado, and D. Wirth (1986). Specific DNA probe for the diagnosis of *Plasmodium falciparum* malaria. *Science* **231**: 117-123.

7. Perler, F.B., and M. Karam (1986). Cloning and characterization of two *Onchocerca volvulus* repeated DNA sequences. Mol. Biochem. Parasitol. **21**: 171-178.

8. McReynolds, L.A., S.M. DeSimone, and S.A. Williams (1986). Cloning and comparison of repeated DNA sequences from the human filarial parasite *Brugia malayi* and the animal parasite *Brugia pahangi*. Proc. Natl. Acad. Sci. USA **83**: 797-801.

9. Williams, S.A., S.M. DeSimone, and L.A. Reynolds (1988). Species- specific oligonucleotide probes for the identification of human filarial parasites. Mol. Biochem. Parasitol. **28**: 163-170.

10. Beaucage, S.L., and M.H. Caruthers (1981). Deoxynucleoside phosphoamidites--a new class of key intermediates for deoxypolynucleotide synthesis. Tetrahedron Lett. **22**: 1859-1862.

11. Maniatis, T., E.F. Fritsch, and J. Sambrook (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, New York.

12. Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, and H.A. Erlich (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**: 487-491.

13. Saiki, R.K., S.J. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim (1985). Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis os sickle cell anemia. Science **230**: 1350-1354.

14. Scharf, S.J., G.T. Horn, and H.A. Erlich (1986). Direct cloning and sequence analysis of enzymatically amplified genomic sequences. Science **233**: 1076-1078.

15. Gyllensten, U.B. and Erlich, H.A. (1988). Generation of single stranded DNA by the polymerase chain reaction and its application to direct sequencing of the HLA DQ alpha locus. Proc. Natl. Acad. Sci. USA. **85**: 7652-7656.

16. Kafatos, F.C., C.W. Jones, and A. Efstratiadis (1979). Determination of nucleic acid sequence homologies and relative concentrations by a dot blot hybridization procedure. Nucl. Acid Res. **7**: 1541-1552.

17. Maxim, A. and W. Gilbert (1980). Sequencing end-labeled DNA with base-specific chemical cleavages. Methods Enzymol. **65**: 499-560.

18. Hsu, S.M., L. Raine, and H. Fanger (1981). A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. **75**: 734-738.

19. Hsu, S.M., L. Raine, and H. Fanger (1981). Use of avidin-biotin- peroxidase complex (ABC) in immunoperoxidase techniques: A comparision between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. **29**: 577-580.

20. Coyle, P.M., G.H. Thorpe, L.J. Kricka, and T.P. Whitehead (1986). Enhanced luminescent quantitation of horseradish peroxidase conjugates: applications in an enzyme immunoassay for digoxin. Ann. Clin. Biochem. **23**: 42-6.

21. Whitehead, T.P., G.H.G. Thorpe, T.J.N. Carter, C. Groucutt, and L.J. Kricka (1983). Enhanced

luminescence procedure for sensitive determination of peroxidase-labeled conjugates in immunoassay. Nature **305**: 158-159.

22. Renz, M., and Kurz Christina (1984). A colorimetric method for DNA hybridization. Nucleic Acid Res. **12**: 3435-3444.