PAPER CHROMATOGRAPHY HYBRIDIZATION: A RAPID METHOD FOR DETECTION OF ONCHOCERCA VOLVULUS DNA AMPLIFIED BY PCR

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Abstract. Prior studies have shown that Onchocerca volvulus DNA can be detected in skin snips and in black flies after polymerase chain reaction (PCR) with primers specific for repeated “O-150” DNA sequences. We have adapted a paper chromatography hybridization assay (PCHA) to detect amplified O-150 DNA and compared this method to two established methods, namely agarose gel electrophoresis (AGE) and hybridization enzyme-linked immunosorbent assay (ELISA). The minimum amounts of purified O-150 DNA detected by PCHA, AGE, and ELISA were 5, 10, and 2 ng, respectively. The three methods had similar estimated sensitivities for detecting O. volvulus DNA amplified from skin snips from African subjects with onchocerciasis (88%, 84%, and 91%, respectively). No false positive results were observed with skin snips from uninfected control subjects. The paper chromatography hybridization assay detects PCR products in 30 minutes without electricity or special equipment. This technology brings DNA detection a step closer to widespread use in field settings.

INTRODUCTION

The parasitic nematode Onchocerca volvulus infects an estimated 18 million people in Africa and Latin America. Onchocerciasis is a major cause of blindness and dermatitis in endemic areas. Attempts to control onchocerciasis have centered on the control of the Simulium fly vectors and mass treatment with ivermectin. Accurate and specific diagnostic methods are needed for identifying endemic areas and for detecting residual or new infections in the wake of control programs.

Traditionally, diagnosis of onchocerciasis has been based on identification of microfilariae (MF) in skin snips by microscopy, an insensitive method. Antibody detection improves sensitivity and avoids the cumbersome and unpopular skin snipping procedure, but this method cannot distinguish between past and current infections. DNA detection has been developed as a definitive diagnostic method for onchocerciasis following the identification of an Onchocerca-specific 150 base pair (bp) DNA sequence (O-150) that is repeated in tandem in O. volvulus genomic DNA. This has enabled the identification of genus-specific primers for polymerase chain reaction (PCR) amplification and development of species and strain-specific DNA probes. Polymerase chain reaction-based assays have been used for detecting O. volvulus DNA in skin snips in central laboratories of the Onchocerciasis Control Program in West Africa and Ecuador.

Methods employed to date for detecting amplified O. volvulus DNA have included agarose gel electrophoresis (AGE), Southern blot followed by hybridization with labeled DNA probes, and a recently developed hybridization/ELISA protocol (PCR-ELISA). In the present study, we have used a new, rapid method to detect O-150 DNA after PCR, namely paper chromatography hybridization (PCHA), and compared this method with AGE and PCR-ELISA for diagnosis of onchocerciasis.

MATERIALS AND METHODS

Sample collection. Skin snips were obtained from the iliac crest of patients with onchocerciasis in O. volvulus-endemic areas of Ghana and Cameroon and from non-endemic controls with a corneoscleral punch biopsy instrument. Microfilariae (MF) were detected by microscopy after skin snips were incubated overnight in RPMI medium. Control skin samples were obtained from healthy people in Cameroon and the United States with no history of exposure to onchocerciasis. Ethical approval for this project, which employed existing specimens collected in prior projects, was provided by the Washington University Human Studies Committee.

Preparation of DNA from skin snips. DNA from skin snips was isolated by a modified proteinase K digestion protocol previously reported by Zimmerman and others. Skin snips were added to 400 μl TE buffer (10 mM Tris/HCL, 1 mM EDTA, pH 7.6) with 5 μl of 10 mg/ml Proteinase K (Sigma Chemical Co., St. Louis, MO) and 5 μl of 10% sodium dodecyl sulfate (SDS, Sigma). After overnight incubation at 56°C, 10 μl of 1M dithiothreitol was added and the samples were heated in a boiling water bath for 30 min and then treated with 3 cycles of freezing and thawing. DNA was precipitated by the addition of 1 ml of 100% ethanol and 4 N sodium acetate. After 1 hr incubation at −70°C and centrifugation at 14,000 g for 15 min, the samples were dried and resuspended in 200 μl of TE buffer.

PCR amplification. Polymerase chain reaction amplification was carried out in a solution containing 67 mM Tris-HCL (pH 8.8), 6.7 mM MgSO₄, 16.6 mM (NH₄)₂SO₄, 10 mM 2-mercaptoethanol; 100 μM each of dATP, dGTP, dCTP, and dTTP; 10 pM of each of two O-150 specific primers described by Zimmerman and others; 2 units of Taq DNA polymerase (Perkin-Elmer, Norwalk, CT); and either 5 pg of O. volvulus genomic DNA (positive control) or 5 μl of DNA isolated from a skin snip (2.5% of total DNA isolated). PCR primer sequences were as follows: O-150 up = 5'-Biotin-GATTYTTCGCGAANARCGC-3' and O-150 down = 5'-GCXRTTAAATXGXXAAATTC-3', where R = A or G; Y = C or T; and X = A, G, C, or T. The reactions were done in a 9600 GeneAmp PCR machine (Perkin-Elmer) and subjected to 40 amplification cycles, each consisting of 30 sec at 94°C, 2 min at 37°C, and 30 sec at 72°C.

Purification of O-150 PCR products. O-150 PCR products amplified from skin snips were purified with QIAquick spin column kit (Qiagen, Valencia, CA) according to the
Detection of O-150 PCR products. The 150 base pair PCR product was detected by three methods.

A. Agarose gel electrophoresis (AGE). Electrophoresis was performed with 1.5% agarose gels with 0.5 μg/ml ethidium bromide and TAE buffer (0.04 M Tris-acetate 1 mM EDTA pH 8.0). 10 μl of PCR product were loaded per lane.

B. PCR-ELISA. Amplified PCR product from skin snips was detected by hybridization enzyme-linked immunoassay as previously described by Nutman and others.5 Briefly, polyvinyl microtiter plates were coated overnight at 37°C with 50 μl of 1 μg/ml streptavidin in coating buffer (0.1 M carbonate/bicarbonate pH 9.6). Five μl of biotin-labeled O-150 PCR product and 15 μl of hybridization buffer (6X SSPE/5X Denhardt’s solution/0.1% Na-Sarcosine/0.02% SDS) were placed in microtiter wells and incubated for 30 min at room temperature. After a denaturing step (1 N NaOH), a fluorescein (FL)-labeled O. volvulus-specific oligonucleotide probe OVS-2 (5'-FL-CCCTAATCTCAAAAAACGGG-FL-3') in hybridization buffer was added to each well for hybridization at 37°C for 30 min. Following high-stringency washing in 20X SSPE/0.1% SDS buffer for 5 min at 37°C (20X SSPE, 0.5 M sodium chloride, 0.23 M sodium phosphate, 0.2 M EDTA), PCR product was detected by ELISA with alkaline phosphatase-anti-fluorescein Fab (AP-α-FL-Fab) (Boehringer Mannheim GmbH, Mannheim, Germany) in TBS/1% BSA buffer (30 min, 37°C) and substrate (BRL ELISA Amplification System, Gaithersburg, MD, 30 min at room temperature). Optical density was read at 670 nm with a microplate ELISA reader.

C. Paper chromatography hybridization assay (PCHA). This test is based on chromatographic migration of amplified biotin-labeled DNA into a nitrocellulose comb. The biotinylated PCR product is trapped by hybridization to a probe that is immobilized on a nitrocellulose comb. The PCHA assay was performed according to manufacturer’s instructions with Universal GeneComb kit reagents (BioRad, Hercules, CA), as shown schematically in Figure 1. Briefly, 0.5 μl of 100 ng/ml OVS-2 probe (or an unrelated control probe) was bound to nitrocellulose membrane with a clear plastic backing by UV cross-linking (500 mj/cm²). Five μl of biotin-labeled PCR product and 5 μl of distilled water were added to small test tubes and denatured with 2 μl of denaturation buffer. After 5 min at room temperature, the solution was neutralized by adding 50 μl of hybridization buffer. The mixtures were transferred into the wells of the first row of a microtiter plate. One drop of streptavidin-alkaline phosphatase conjugate, chromogenic substrate, and a stop solution were added to wells in the second, third, and fourth rows of
DETECTION OF ONCHOCERA VOLVULUS DNA

FIGURE 2. Detection of *Onchocerca volvulus* DNA in skin snips by paper chromatography hybridization assay (PCHA). Gray spots (developed over immobilized probe placed in duplicate on each tooth of the comb) indicate a positive test. The figure illustrates weak and strong positive tests. The specimen tested in the third tooth from the right (#51) was a negative control.

the plate, respectively. The nitrocellulose GeneComb with immobilized OVS-2 probe was then inserted into the wells of the first row of the microtiter plate for hybridization and maintained at 37°C for 15 min. The teeth of the comb were then placed sequentially (without washing steps) in the second, third, and fourth rows of the microtiter plate for 5 min, 7 min, and 3 min, respectively at room temperature (Figure 2). Positive reactions (binding of amplified O-150 DNA to the OVS-2 probe) produced a blue spot over the immobilized probe. The test was scored negative if no blue spot appeared.

**Statistical analysis.** Skin snips with MF observed by microscopy were considered to be truly positive, and the operational sensitivity of DNA tests was taken to be the percentage of positive skin snips that were positive by DNA detection. The significance of differences in sensitivity observed with the three DNA detection methods was assessed by chi-square. In contrast to operational sensitivity, the absolute sensitivity of methods for detection of O-150 PCR products was defined as the minimum amount of purified O-150 PCR product detected by each method.

**RESULTS**

**Detection of purified O-150 PCR product.** Serial dilutions of the purified O-150 PCR product were tested by AGE, PCR-ELISA, and PCHA. The minimum amounts of PCR-amplified product detected by these methods (absolute sensitivity) were 10 ng, 2 ng, and 5 ng, respectively.

**Detection of *O. volvulus* DNA in human skin snips.** The sensitivity of AGE and PCHA relative to ELISA was also assessed with PCR products amplified from skin snips. Most specimens were strongly positive by all methods, so the comparative study was performed with samples with weak-to-moderate signals by ELISA to highlight differences (Table 1). These studies showed that PCHA was usually positive when ELISA produced optical density (OD) values ≥0.3, while AGE was positive when ELISA OD values exceeded 0.5. Both PCHA and AGE were uniformly negative when ELISA OD was <0.2. Note that OD values <0.2 were considered as negative by ELISA. This information was used to estimate the relative operational sensitivity of the three methods for detecting *O. volvulus* DNA with a panel of 133 skin snips from MF-positive onchocerciasis patients. The estimated operational sensitivities of ELISA, PCHA, and AGE for these samples were 91%, 88%, and 84%, respectively (Table 2). These differences in sensitivity were not significant by chi-square. No false positive results were obtained by any of the methods with PCR products amplified from skin samples from people who had not been exposed to onchocerciasis.

Microfilaria counts in the samples tested ranged from 0.3

<table>
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<th>Table 1</th>
<th>Comparison of three polymerase chain reaction-based methods for detection of <em>Onchocerca volvulus</em> DNA</th>
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<td>ELISA (OD)</td>
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<td>&lt; 0.2</td>
<td>0/12</td>
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<td>0.2–0.29</td>
<td>1/6</td>
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<td>0.3–0.5</td>
<td>10/10</td>
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<td>&gt; 0.5</td>
<td>21/22</td>
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<td>ELISA = enzyme-linked immunosorbent assay.</td>
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<td>OD = optical density; OD values &lt;0.2 were considered to be negative by ELISA.</td>
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<td>PCHA = paper chromatography hybridization assay.</td>
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<td>AGE = agarose gel electrophoresis.</td>
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<td>MF = geometric mean of microfilariae per skin snip.</td>
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<th>Table 2</th>
<th>Estimated sensitivity of three polymerase chain reaction-based methods for detecting <em>Onchocerca volvulus</em> DNA in skin snips from microfilaria-positive subjects</th>
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<tr>
<td></td>
<td>No. positive</td>
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<tr>
<td>AGE</td>
<td>112</td>
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<tr>
<td>PCR-ELISA</td>
<td>121</td>
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<tr>
<td>PCHA</td>
<td>117</td>
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<tr>
<td>AGE = agarose gel electrophoresis.</td>
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to 400 per skin snip (median 46.7). There was no significant correlation between skin microfilaria counts and optical densities in the PCR-ELISA test (r = 0.094, P > 0.05).

DISCUSSION

A variety of methods has been used to detect parasite DNA in clinical specimens or in insect vectors. Progress has been slower in modifying these methods to permit widespread use of DNA detection in laboratories in disease-endemic countries. Onchocerciasis is a special example of this general pattern. Prior studies have shown that PCR with primers that amplify O-150 repeat DNA can be used to detect *O. volvulus* DNA in skin snips from patients with onchocerciasis and in infected *Simulium* flies. To our knowledge, DNA detection has been successfully performed in only two laboratories in disease-endemic countries—in Ecuador and in the central laboratory of the Onchocerciasis Control Program in West Africa. Improved and simplified methods will be needed before DNA detection can be widely used in developing countries where parasitic diseases are important causes of morbidity and mortality. The present study focused on one aspect of the problem, namely detection of amplified parasite DNA following PCR.

Three methods for detection of PCR product were compared using previously described methods for DNA isolation and for amplification of the O-150 target sequence. Agarose gel electrophoresis was the least sensitive detection method tested in terms of absolute sensitivity, although its operational sensitivity (% positive) between PCHA, AGE, or ELISA for detecting O-150 DNA amplified from skin snips from people with onchocerciasis. Thus, the operational sensitivity of these assays with the skin snips we tested was equivalent. Most subjects in our series had high skin microfilaria counts; different results might have been observed if more of the snips tested had been from subjects with low intensities of infection. Hybridization ELISA and PCHA are similar in concept; the absolute sensitivity and specificity of these methods are enhanced compared to AGE by use of the species-specific OVS-2 probe, which was designed on the basis of extensive sequence data from *O. volvulus* and related species. The main advantages of PCHA over DNA hybridization ELISA are speed and simplicity. Paper chromatography hybridization assay detects amplified O-150 DNA in 30 minutes without requiring electricity or the equipment needed for ELISA. Thus, PCHA could be performed in field laboratories that would not be able to perform DNA-hybridization ELISA. This technology brings DNA detection a step closer to being practical for widespread use in developing countries. However, additional work will be needed to simplify methods for DNA isolation and amplification of target DNA to make detection of microbial DNA a practical option for laboratories in developing countries.

Previous studies have shown that the ELISA-based PCR detection method employed in this study can produce a positive signal with as little as 2.5 fg of plasmid-derived O-150 DNA template estimated to be equivalent to 300 fg of genomic DNA for *O. volvulus*. The present study showed that PCR-ELISA was the most sensitive method tested for detection of purified O-150 PCR product. However, this assay is labor-intensive and it takes two days to perform. It also requires expensive reagents and equipment, such as an ELISA reader for optimal performance. Thus, while PCR-ELISA is very sensitive and has been successfully performed in laboratories in onchocerciasis-endemic countries, it is well beyond the capabilities of most laboratories in developing countries.

The lack of correlation between skin microfilaria counts and optical densities in the PCR-ELISA assay is not surprising because the PCR protocol was designed for optimal sensitivity as a qualitative test. If desired, it should be possible to modify the assay for quantitation of template DNA.

The absolute sensitivity of PCHA for detecting purified O-150 DNA was intermediate between those of AGE and ELISA. However, there was no significant difference in operational sensitivity (% positive) between PCHA, AGE, or ELISA for detecting O-150 DNA amplified from skin snips from people with onchocerciasis. Thus, the operational sensitivity of these assays with the skin snips we tested was equivalent. Most subjects in our series had high skin microfilaria counts; different results might have been observed if more of the snips tested had been from subjects with low intensities of infection. Hybridization ELISA and PCHA are similar in concept; the absolute sensitivity and specificity of these methods are enhanced compared to AGE by use of the species-specific OVS-2 probe, which was designed on the basis of extensive sequence data from *O. volvulus* and related species. The main advantages of PCHA over DNA hybridization ELISA are speed and simplicity. Paper chromatography hybridization assay detects amplified O-150 DNA in 30 minutes without requiring electricity or the equipment needed for ELISA. Thus, PCHA could be performed in field laboratories that would not be able to perform DNA-hybridization ELISA. This technology brings DNA detection a step closer to being practical for widespread use in developing countries. However, additional work will be needed to simplify methods for DNA isolation and amplification of target DNA to make detection of microbial DNA a practical option for laboratories in developing countries.

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