

SPECIES-SPECIFIC SEQUENCE IN THE REPEAT 3 REGION OF THE GENE ENCODING A PUTATIVE *LOA LOA* ALLERGEN: A DIAGNOSTIC TOOL FOR OCCULT LOIASIS

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Abstract. A polymerase chain reaction (PCR)-based method to detect *Loa loa* DNA in the blood lysate of infected individuals is described. A set of primers was designed to amplify the repeat 3 sequence (15r3) of the gene encoding a putative *L. loa* allergen. The qualitative PCR was carried out using blood lysates from subjects from an *L. loa*-endemic area of Gabon where loiasis exists sympatrically with *Mansonella perstans*, and from individuals from a loiasis-free area in Togo infected concomitantly with *M. perstans* and *Onchocerca volvulus*. No specific amplification was observed after ethidium bromide staining of a gel containing *M. perstans* and *O. volvulus* control samples. In contrast, a 396-basepair (bp) DNA was detected in all *L. loa* microfilaremic individuals and in seven of the 20 *L. loa* amicrofilaremic subjects diagnosed by leukoconcentration. Qualitative Southern blots carried out at high stringency (65°C) using 15r3 oligonucleotide probe revealed hybridization only with *L. loa* samples (5 of 5 microfilaremic individuals and 15 of 20 amicrofilaremic individuals), confirming the results obtained with ethidium bromide staining of PCR products. We conclude that this 396-bp sequence could be used as a species-specific diagnostic tool for occult loiasis in an endemic area with concurrent filarial infections.

Loa loa is a human filarial parasite transmitted by *Chrysops dimidiata* and *C. silacea* of the family Tabanidae. The vector, a diurnal biting fly, is limited to the rain forest of Central and West Africa. The common symptoms associated with this infection are pruritus, Calabar edema, and inflammation of the conjunctiva, with ocular passage of the adult worm.¹ In loiasis-endemic areas, microfilariae are typically detected in fewer than 30% of the individuals.² However, seroepidemiology studies demonstrated that 80% of amicrofilaremic individuals in an endemic area have antibodies against *L. loa* antigens.³ Human loiasis is currently diagnosed by the detection of microfilariae in the blood by leukoconcentration, by observation of the ocular passage of the adult worm, or by the observation of suggestive clinical signs. These methods are insensitive and can only roughly estimate the true prevalence. Various antibody-based assays have also been used to measure host antibodies in *L. loa*-exposed or -infected individuals. However the major limitation of serologic tests of filariasis such as loiasis is their low specificity due to the cross-reactivity among filarial antigens. The identification of the filarial species-specific DNA sequences that can be detected in infected blood and amplified by the polymerase chain reaction (PCR), using specific primers, may overcome these problems of nonspecificity. We investigated the species specificity of the repeat 3 sequence of the gene encoding a putative *L. loa* allergen, which is detectable in blood lysates of *L. loa*-infected individuals using the PCR.

MATERIALS AND METHODS

Blood collection. The Gabonese subjects were 25 individuals living in the village of Okoumbi, located 50 km from Franceville, in an area of Gabon where *L. loa* is found sympatrically with *Mansonella perstans*. Blood samples were collected by venipuncture into vacutainers containing EDTA and transported on ice to the Centre International de Recherches Medicales de Franceville (CIRMF), where 100- μ l aliquots were frozen at -80°C for the PCR. The leukocon-

centration method with saponin was used for enumeration of blood microfilariae. The distinction between *L. loa* and *M. perstans* microfilariae was based on size, motility, and the presence or absence of the sheath. Twelve blood samples were collected from residents of Sagbadai, a village near Sokode, Togo, an area endemic for onchocerciasis and mansonellosis, but not loiasis. Blood samples from these subjects were collected in vacutainers containing EDTA and transported in liquid nitrogen from Togo to the CIRMF in Gabon.

Leukoconcentration. Blood was collected by venipuncture into tubes containing EDTA. One milliliter of each sample was diluted in 15-ml tubes containing 9 ml of phosphate-buffered saline in duplicate. The mixture was treated with 600 μ l of 2% saponin at room temperature for 15 min to lyse the red blood cells and centrifuged at 1,000 $\times g$ for 15 min at 4°C. The supernatant was discarded and the pellet was examined microscopically for microfilariae.

Extraction of DNA. For each sample, 100 μ l of whole blood was mixed with 500 μ l of TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), centrifuged at 10,000 $\times g$ for 2 min, and the supernatant was discarded. This step was repeated and the pellet was resuspended in 500 μ l of sucrose buffer (10 mM Tris, pH 7.6, 5 mM MgCl₂, 1 M sucrose, and 1% Triton X-100) and centrifuged twice as above. After a final wash in sucrose buffer, the supernatant was discarded and the pellet was resuspended in 200 μ l of prewarmed (56°C) proteinase K buffer (20 mM Tris, pH 8, 50 mM KCl, 2.5 mM MgCl₂, 0.5% Tween 20) plus 100 μ g/ml of proteinase K (Boehringer-Mannheim, Mannheim, Germany). Incubation at 56°C for 2 hr was followed by heating at 90°C for 10 min. The DNA can be stored at 4°C for several days or at -20°C for at least two years.

Polymerase chain reaction. Based on the repeat 3 sequence of the gene coding for *L. loa* 15-kD antigen, primers corresponding to the extreme 5' and 3' region of this repeat 3 region were synthesized at CIRMF. The sequences of these primers are as follows: forward primer (24-mer): 5'-AAT-CAG-GCA-AAT-AAT-GGC-ACA-AAA-3', reverse primer (24-mer): 5'-GCG-TTT-TCT-TCT-CAC-CAG-CTG-TCT-3'.

We also synthesized a 27-mer oligonucleotide (5'-TCA-AGA-GCC-GAT-ATA-CTG-AAA-GCT-ATC-3') that was homologous to an internal sequence of the repeat 3 region and was used as a hybridization probe. Two microliters of each blood lysate were amplified using a Perkin-Elmer (Norwalk, CT) thermal cycler in a 50- μ l volume containing 1 $\times$ PCR buffer (Stratagene, Montigny Le Bretonneux, France) as supplied by the manufacturer (200 mM Tris-HCl, pH 8.7, 100 mM KCl, 100 mM (NH₄)₂SO₄, 20 mM MgSO₄, 1% Triton X-100, 1 mg/ml of bovine serum albumin), 200 μ M each of dATP, dCTP, dGTP, and dTTP, 1 μ M of each primer, and 2.5 units of cloned *Pyrococcus furiosus* (Pfu) DNA polymerase (Stratagene). Amplifications were carried out as follows: a hot start cycle of 96°C for 10 min, 80°C for 5 min, and 94°C for 30 sec, followed by 40 cycles at 94°C for 1 min (denaturation), 65°C for 1 min (annealing), and 72°C for 2 min (extension).

Analysis of PCR products. After amplification, 15 μ l of each PCR product were mixed with 1 μ l of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 40% w/v sucrose in water) and analyzed by electrophoresis on 1.5% agarose gels. The gels were stained with ethidium bromide and the DNA was visualized and photographed under ultraviolet light. The DNA was then transferred onto Zeta Probe membranes (Bio-Rad Laboratories, Richmond, CA). Prehybridization and hybridization were done respectively at 50°C and 65°C using a buffer containing 75 mM sodium citrate (SSC), 20 mM NaH₂PO₄, 7% sodium dodecyl sulfate (SDS), 10 $\times$ Denhardt's solution, and 100 μ g of denatured herring sperm DNA. The oligonucleotide probe was labeled with α -³²P-dCTP using terminal transferase (Stratagene) according to the manufacturer's recommendations. The membranes were washed after hybridization at 65°C using wash 1 (45 mM SSC, 10 $\times$ Denhardt's solution, 5% sodium dodecyl sulfate, and 25 mM NaH₂PO₄) for 30 min and wash 2 (15 mM SSC, 1% SDS) for 30 min. Hybridizing bands were revealed by autoradiography with x-ray film (Eastman-Kodak, Rochester, NY). The radiographs were developed after exposures of 4 hr to one week.

RESULTS

Thirty-seven blood samples were analyzed: 12 samples collected in Togo and 25 samples collected in an *L. loa*-endemic area of Gabon (Table 1). Of the 25 samples from Gabon, five were positive for the presence of *L. loa* microfilariae (classified as mf+ and 20 were negative [mf-]) by microscopic examination. Twelve of the 20 *L. loa* mf- samples were found to carry *M. perstans* microfilariae, whereas four of the five *L. loa* mf+ samples also had *M. perstans* microfilariae. All samples from Togo showed *M. perstans* microfilariae on microscopic examination. Each of these blood samples was processed as described in the Materials and Methods. Figure 1A represents a typical electrophoretic profile of the PCR products. *Loa loa* mf+ samples or recombinant plasmid DNA (carrying the *L. loa* 15 kD gene) showed a strong signal at the expected band 396-basepair (bp) level (stained with ethidium bromide) and few nonspecific bands. In contrast, only seven of the 20 *L. loa* amicrofilaremic samples were positive by ethidium bromide staining and showed a very faint band at 396 bp and multiple

TABLE 1
Results of gold standard microscopy compared with those of polymerase chain reaction/hybridization

Sample no. and origin*	Blood microfilariae		Ethidium bromide staining of the gels	Southern hybridization
	<i>Loa loa</i> /ml	<i>Mansonella perstans</i> /ml		
T1	0	14	—	—
T2	0	4	—	—
T3	0	6	—	—
T4	0	41	—	—
T5	0	16	+	—
T6	0	10	—	—
T7	0	60	—	—
T8	0	34	—	—
T9	0	20	—	—
T10	0	8	—	—
T11	0	24	—	—
T12	0	32	—	—
G28	0	0	—	—
G30	0	210	—	—
G237	0	0	—	—
G243	0	989	—	—
G244	0	3	—	—
G21	0	4	—	+
G22	0	404	—	+
G23†	0	60	—	+
G25	0	24	+	+
G26	0	0	—	+
G29†	0	11	—	+
G231‡	0	15	+	+
G233	0	0	—	+
G235	0	3	+	+
G236	0	0	—	+
G238	0	34	+	+
G239	0	0	+	+
G240	0	42	+	+
G242	0	0	+	+
G245†	0	0	—	+
G234	12	453	+	+
G27†	90	144	+	+
G24	104	0	+	+
G232	709	205	+	+
G241	3,260	1,120	+	+

* T = sample from Togo; G = sample from Gabon.

† Individuals with angioedema (Calabar edema).

‡ Amicrofilaremic individuals with verified ocular passage of a *L. loa* adult worm.

nonspecific bands. Eleven control samples from Togo did not show any bands of the expected size; however, one sample showed a faint band at 396 bp. To test the species-specificity of the PCR assay, Southern blot hybridization at high stringency (65°C) was performed using a 15r3 oligonucleotide probe labeled as described above. Hybridization was observed only with the samples collected from the loiasis-endemic area (Figure 1B). All *L. loa* microfilaremic samples and 13 of the 20 *L. loa* amicrofilaremic samples were positive on hybridization after a 48-hr exposure to the film. Two amicrofilaremic samples were positive after an exposure of one week. The sample from Togo that was stained by ethidium bromide was negative in the Southern blot.

DISCUSSION

The gene encoding a putative *L. loa* allergen has been characterized using an *L. loa* genomic DNA library.⁴ It is 1,761 bp in length, contains five repeated sequences, and codes for a putative 15-kD *L. loa* antigen. The repeat 3

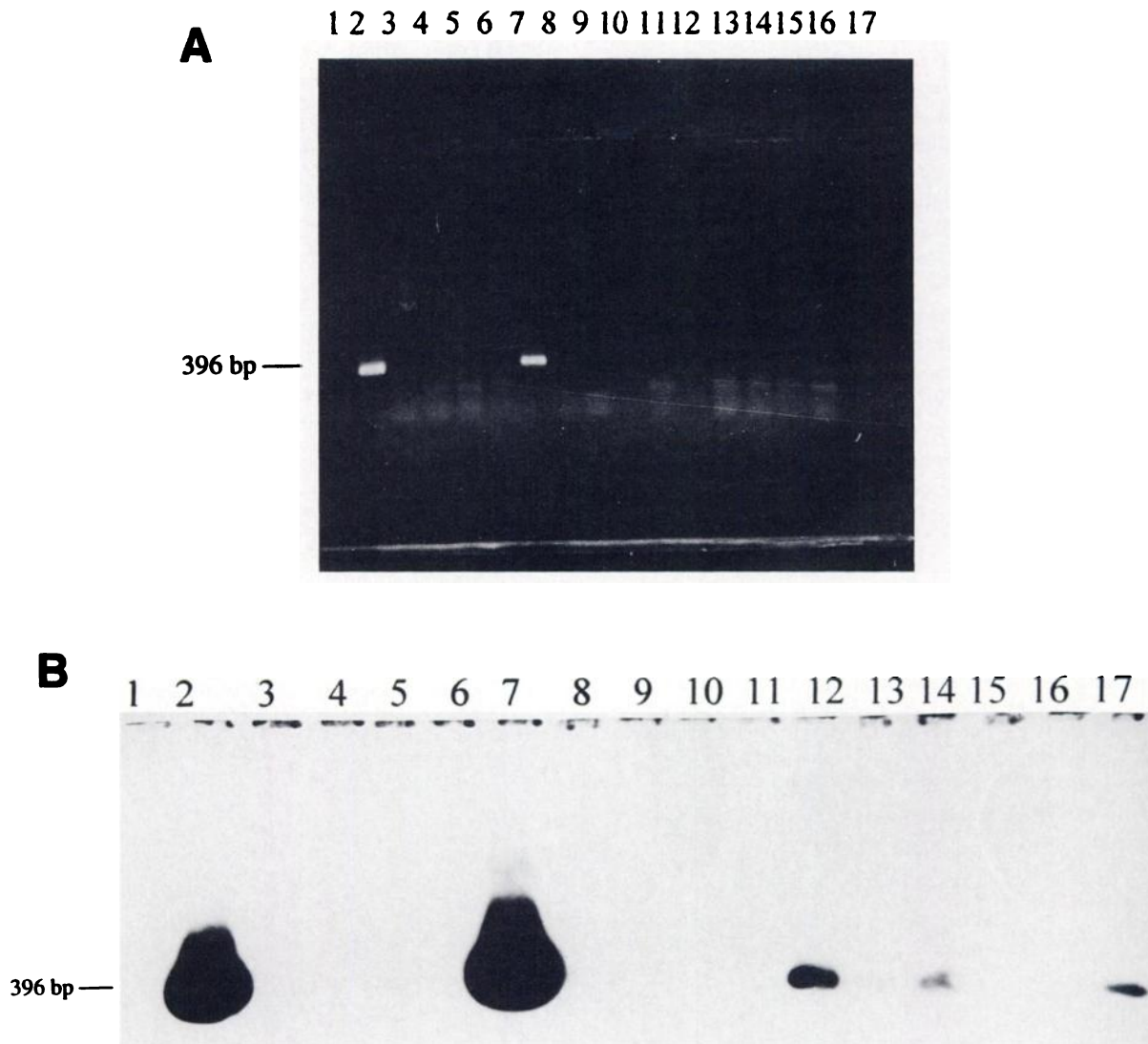


FIGURE 1. **A**, representative electrophoretic profiles of the 15r3 polymerase chain reaction (PCR) products. Six *Loa loa* amicrofilaremic individuals are compared with eight of the 12 *Mansonella perstans* controls. Lane 1, *L. loa*-negative control; lanes 2 and 7, *L. loa* microfilaremic individuals G24 and G232, respectively; *M. perstans* controls: lane 3, T1; lane 4, T2; lane 5, T3; lane 6, T4; lanes 8, T9; lane 9, T10; lane 10, T11, lane 11, T12; lanes 12–17, *L. loa* amicrofilaremic individuals G235, G236, G238, G239, G240, and G242, respectively. bp = basepairs. **B**, specificity of the 15r3 PCR assay. Southern blot of samples in A; all *L. loa* amicrofilaremic individuals (lanes 12, 13, 14, 15, 16, and 17) and *L. loa* microfilaremic individuals (lanes 2 and 7) show a band at 396 bp. Possibly due to differential parasite loads, the bands in lanes 13, 15, and 16 were apparent in the radiograph but too faint to be seen in the photograph. The PCR negative control (lane 1) and the eight *M. perstans* controls (lanes 3, 4, 5, 6, 8, 9, 10, and 11) show no bands. The film was developed after an exposure of one week.

(15r3) sequence of this gene is 396 bp in length, is not G + C rich, and is detectable in blood lysates of *L. loa*-infected individuals by the PCR. We tried to determine the specificity of this 15r3 sequence for *L. loa* by comparative analysis of qualitative PCR products using blood lysates from individuals in an *L. loa*-endemic region and from *Onchocera volvulus*-plus *M. perstans*-infected subjects.

Twenty of 25 samples collected in a loiasis-endemic area of Gabon were positive by Southern blotting. Hybridization-positive samples showed bands at 396 bp indicating that these PCR amplification products are, in fact, the *L. loa* 15r3 repeat. The Southern blots carried out at high stringency (65°C) showed no hybridization with *M. perstans* and *O. volvulus* samples after an exposure of one week to the x-ray film, indicating that the 15r3 PCR assay is species-specific

for *L. loa*. There was no PCR contamination since the PCR-negative control lanes (no template DNA) had no bands.

The results of this study show that 15 of 20 *L. loa* amicrofilaremic samples were positive in the Southern blot hybridization and suggest that these individuals carried an infection that could not be detected by standard techniques. It is likely that circulating *L. loa* DNA, released by lysed microfilariae or from the adult worm, was detected in these cases. The possibility of detecting circulating DNA released from dead filariae by the PCR has also been suggested for infection with *Brugia malayi*.⁵ The five *L. loa* amicrofilaremic individuals, who were Southern hybridization-negative, represent the putative resistant subjects. Two repetitive DNA sequences that proved to be specific for *L. loa* have been reported.^{6,7} One of these repetitive sequences was used

as a DNA probe to detect a single *L. loa* microfilaria, and its usefulness in identifying *L. loa* microfilariae in the blood of patients with mixed filarial infections and in the rapid screening of large number of microfilaremic samples has also been reported.⁶ Nevertheless, this previous study did not demonstrate clearly the ability to detect occult *L. loa* infection. Perhaps the most significant finding of this present study is that occult loiasis, which is the most common form in a loiasis-endemic area, can be detected using this 15r3 PCR assay.

This initial study was based on a small, random sample of 25 individuals, only five of whom were found (on standard microscopy examination) to be infected with *L. loa*. Despite the small sample size, the initial data suggest that this assay could be useful in detecting *L. loa* infection. Further studies will be performed to evaluate the sensitivity of this 15r3 PCR assay in detecting occult infections, i.e., in amicrofilaremic individuals with verified ocular passage of a *L. loa* adult worm.

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