SENSITIVE AND SPECIFIC ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DIAGNOSIS OF WUCHERERIA BANCROFTI INFECTION IN URINE SAMPLES


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Abstract. We developed an enzyme-linked immunosorbent assay (ELISA) that detects filaria-specific immunoglobulin G4 antibodies in unconcentrated urine. The ELISA was positive in 87 of 91 (95.6%) urine samples collected from people with Wuchereria bancrofti microfilariae, antigen, or both. Of 298 urine samples collected in Thailand, Lao People’s Democratic Republic, and Japan, where no human filariasis is known, 95 (99.0%) were negative by ELISA. Various intestinal nematode and fluke infections did not interfere with the ELISA. Urine samples with sodium azide could be kept at 37°C for 4 weeks, and the time of urine collection did not influence ELISA results. This ELISA can be used to identify endemic foci of filariasis.

INTRODUCTION

In May 1997, the 50th World Health Assembly made a resolution to eliminate lymphatic filariasis as a public health problem worldwide. The basic strategy is to treat everyone in endemic areas with a single dose of antifilarial drugs annually for 4–6 years. One of the prerequisites for treatment is to have information on possible endemic foci. To identify endemic foci and delineate areas to be treated as effectively as possible, several rapid assessment procedures have been used. The rapid assessment procedures include 1) recording obvious clinical signs of filariasis such as hydrocele or elephantiasis, 2) detecting circulating filarial antigen by means of a simple card test, and 3) finding infected vectors by detecting filarial DNA in mosquitoes.

Recording clinical cases is simple but not very accurate in terms of recent infection. The card test based on immunochromatography is easy, quick, and accurate, but it is expensive and requires pricking the finger to obtain a blood sample, which people usually dislike. The presence of infected mosquitoes is an indicator of human infection, but the use of polymerase chain reaction is limited to well-equipped laboratories. The latter two procedures are most useful in known areas of endemic filariasis to assess the level of endemicity and prioritize areas for treatment. To identify new foci, filarial antibody detection has the advantage of higher prevalence than antigen detection. Antibody detection is also useful in confirming the absence of filariasis in areas not previously surveyed.

To facilitate sample collection for immunodiagnosis, urine samples have been used to test for schistosomiasis, malaria, leishmaniasis, Chagas disease, and filariasis. In human filariasis, the usefulness of immunoglobulin (Ig) G4 in the diagnosis of filarial infections has been reported.

In this study, we report a new sensitive and specific enzyme-linked immunosorbent assay (ELISA) that detects filaria-specific IgG4 in urine samples.

MATERIALS AND METHODS

Study subjects. In Matara, Sri Lanka, where nocturnally periodic Wuchereria bancrofti is endemic, people were examined for microfilariae by Nucleopore filtration of 1 mL of venous blood or by 60 μL fingerprick blood smear, and for filarial circulating antigens with Og4C3 ELISA kit (JCU Tropical Biotechnology Pty. Ltd., Queensland, Australia) between 10 PM and 2 AM. On the basis of the results, urine samples were collected from 117 people, 43 of whom were positive for the antigen and microfilariae (Ag+/Mf+), 45 were positive for the antigen only (Ag+/Mf−), 3 were positive for microfilariae only (Ag−/Mf+), and 26 were negative both for the antigen and microfilariae (Ag−/Mf−).

In Laos and northeastern Thailand, where filariasis is unknown, stool samples were examined by the Kato-Katz method in the former and by formalin-ether concentration technique in the latter; egg-positive subjects were asked for urine samples. Of 70 samples collected, 23 were positive for Ascaris lumbricoides, 8 for hookworm, 24 for Opisthorchis viverrini, 7 for Echinostoma sp., 1 for Strongyloides stercoralis, and 12 for unidentified minute intestinal flukes. There were also 5 samples with 2 different species of eggs, for a total of 75 egg-positive patients. All the people infected with Ascaris were from Laos. Additional urine samples from nonendemic areas were examined, including 186 samples from nursing students in Bangkok and 42 from Japanese university students. Sodium azide was added to all urine samples at a final concentration of ~0.1%.

The blood tests in Matara were carried out with an approval by the ethics committee of the Faculty of Medicine, University of Ruhuna, and all urine samples were collected after the purpose of study had been explained and informed consent obtained.

Enzyme-linked immunosorbent assay. Antigens were prepared from adult Brugia pahangi female worms collected from the abdominal cavity of Mongolian gerbils. Worms were homogenized in 1/15 M phosphate-buffered saline (PBS), pH 7.4, containing protease inhibitors: 1 mM of phenylmethylsulfonyl fluoride, 10 μM of [l-3-trans-carboxyoxiran-2-carbonyl]-l-leucyl-agmatin (E-64), 0.5 mM of Pepstatin A, and 5 mM of ethylenediaminetetraacetic acid. The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C, and the supernatant was stored at −40°C until use.

The IgG4 ELISA for detecting antibodies was carried out...
as follows. A 96-well microtiter plate was coated with B. pahangi antigens (5 μg/mL) at 4°C overnight. The surface of the plate was blocked with casein buffer (1% casein in 0.05 M Tris-HCl buffer with 0.15 M NaCl, pH 7.6) for 2 hr at room temperature. Urine samples were directly applied to the plate (100 μL per well) and incubated overnight at 25°C. The plate was washed three times with Tween-PBS (0.05% Tween 20 in 1/15 M PBS, pH 7.4), and 100 μL peroxidase-conjugated mouse monoclonal antibody to human IgG4 (Caltag Laboratories Inc., San Francisco, CA), diluted 1:1,000, was added to each well. After incubation at 37°C for 1 hr, the plate was washed three times, ABTS peroxidase substrate (KPL Inc., Gaithersburg, MD) was added, and absorbance was measured at 415 nm.

Antibody levels were expressed as units on the basis of a standard curve. To construct the curve, pooled sera from 5 patients with bancroftian filariasis were serially diluted 3-fold (1:3,000 to 1:2,187,000) with casein buffer supplemented with sodium azide, and a set of the serially diluted sera was prepared for each microtiter plate. As antibody units, a value of 7,290 U was arbitrarily assigned to the 1:3,000 dilution and a value of 10 U to the 2,187,000 dilution. The average antibody unit was computed as a geometric mean of (antibody unit + 1). Antibody units >7,290 U were regarded as 7,290 U.

Effects of storage of urine samples and timing of sample collection on antibody units. To study how long urine samples could be kept without deterioration at ambient temperatures, urine was kept in an incubator at 25°C or 37°C for up to 4 weeks and the change in antibody units was examined weekly. Fifteen samples (5 with high units, 5 with lower units, and 5 with negative findings) were used for each temperature.

Ten antibody-positive people were requested to collect urine samples early in the morning, at 11 AM–12 AM, and at 4:30 PM–5:00 PM. The antibody units and the fluctuation by time were studied.

RESULTS

Urine samples from a total of 117 Sri Lankans from Mata, 186 Bangkok students, 70 northeastern Thai and Lao residents, and 42 Japanese students were examined for IgG4 antibodies (Figure 1). Among control groups, northeastern Thai and Lao people combined had a higher average antibody unit (4.7 U) than Bangkok students (2.0 U) (t-test, P < 0.01), and Japanese students had a lower average (1.6 U) than Bangkok students (t-test, P < 0.03). In this study, the cutoff value was defined as the average unit of all Thai and Lao people ± 3 standard deviations, which was 54.7 U. Of 91 filaria-infected people, 87 (95.6%) were antibody positive. There were no differences in the average antibody unit (1,294 versus 1,178 U; t-test, P > 0.7) or sensitivity (97.7 versus 93.3%; chi-square test, P > 0.3) between Ag+/Mf+ and Ag+/Mf− people. Fifty-four percent of endemic normals (Ag−/Mf−) were also positive. In contrast, 295 of 298 people (99.0%) from the nonendemic areas were antibody negative.

Antibodies are analyzed in Figure 2 according to the species of infected parasites. Three false-positive findings were found, one each in hookworm, minute intestinal flukes, and Echinostoma infections, and their antibody units were much lower compared with filaria-infected people. We conclude that ELISA that uses urine has high sensitivity and specificity, and that cross-reactivity with common intestinal parasites in this part of Asia is minimal.

To determine the effect of storage, antibody units were measured in urine samples kept at 25 or 37°C up to 4 weeks. The result at 37°C is shown in Figure 3. One sample with a relatively high antibody unit showed a clear decrease in the value, but as a whole, antibody levels were stable during this period. No sample turned from positive to negative. At 25°C, the unit values were more stable than at 37°C (data not shown). The effect of timing of urine collection on antibody units was studied in 10 antibody-positive patients (Figure 4). The fluctuation was small, and all samples remained positive for any time of the day.
Figure 3. Effect on antibody units of keeping urine samples at 37°C.

Figure 4. The fluctuation of antibody units by time of urine collection.

DISCUSSION

Filarial antigen detection in blood has been established as the most reliable method of determining *W. bancrofti* infection. The method is applicable any time of the day, regardless of the periodicity of microfilaraemia.\textsuperscript{20,21} It is a rapid test, especially when the ICT card test is used,\textsuperscript{4} and it is more sensitive than conventional parasitological methods. On the other hand, antibody detection is less popular for diagnosis of filariasis, mainly because it cannot distinguish between previous and current infections and does not reflect parasite load or pathology. However, for the purpose of finding new endemic foci or to confirm the absence of infection, antibody detection is advantageous because it detects a higher prevalence than antigenemia. By sampling younger people, antibody tests can identify recent infections.

The current study revealed that IgG4 ELISA that used urine samples was sensitive (95.6%) and specific (99.0%) in detecting *W. bancrofti* infection. It was also not necessary to concentrate urine before the assay. This may relate partially to renal damage caused by filarial infection and resultant proteinuria.\textsuperscript{22} The sensitivity was not very high, but judging from the observation that more than half of the endemic normal people were positive, the ELISA will give a much higher prevalence than the antigen assays in a community-based survey.

Immunoglobulin G4 was reported to cross-react with other filarial species such as *Onchocerca volvulus* and *Loa loa*,\textsuperscript{19} and cross-reaction with *Dirofilaria* also has to be considered. In Thailand, *Dirofilaria immitis* infection among dogs is common. In Khon Kaen, an infection rate of 52.5% was reported in 1996 (Chotirosniramit C and Thaiklar K, unpublished data), and a survey in Bangkok in 1998 revealed a rate of 19.0% (Anantaphruti MT, unpublished data). Of 233 Thai people included as negative controls, only 3 false-positive findings with low antibody units were found. This suggests that infections of dog filariasis did not affect the current study. The usefulness of this ELISA in areas with *Onchocerca* or *L. loa* infection is unknown. The secretion or leak of IgG4 into urine would relate to renal pathology, which is probably dependent on the species of infecting parasite.

In immunodiagnosis of parasitic infection, urine has mostly been used for antigen detection. The merit of the use of urine instead of serum is the ease in collecting samples, which will facilitate compliance of people in field activities. Untrained people could collect many samples in a relatively short time—at schools, for example. In this ELISA, samples can be collected any time during the day and can be kept at least for 4 weeks at 37°C, which has practical importance in the field.

Whether IgG4 antibodies in urine can be used for follow-up studies after drug treatment needs further investigation. With serum samples, antibodies of IgG4 subclass were reported to be associated with active filarial infection\textsuperscript{23} or microfilaraemia\textsuperscript{24} and decreased rapidly after treatment.\textsuperscript{25} In patients with *B. malayi*, diethylcarbamazine reduced IgG4 levels by 65–78% in 12 months.\textsuperscript{26} It is also interesting to study if more exposure to infective bites can induce IgG4 antibodies.\textsuperscript{27} A preliminary study in Matara (unpublished data) revealed some very young children who were urine IgG4 positive but ICT test negative, suggesting that antibody would be detected before the antigen. Further studies are needed to confirm this.

No antigen assay system for *Brugia malayi* currently exists, and a rapid assessment procedure for this species has been sought. Because the antigens used in this study were from *B. pahangi* adults, it is worthwhile to test the ELISA for brugian filariasis.

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