Confirmation of Elimination of Lymphatic Filariasis by an IgG4 Enzyme-Linked Immunosorbent Assay with Urine Samples in Yongjia, Zhejiang Province and Gaoan, Jiangxi Province, People’s Republic of China

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Abstract. A sensitive and specific IgG4 enzyme-linked immunosorbent assay (ELISA) with urine samples has been reported. To confirm elimination of bancroftian filariasis, the ELISA was used in a study conducted in Yongjia County and Gaoan City, People’s Republic of China, where filariasis elimination was declared, with 10,409 students 5–16 years of age. The antibody positive rates were 0.08% in Yongjia and 0.34% in Gaoan. All positive samples were re-examined and found to be negative. Our results show that this ELISA is practical and useful for confirmation of the elimination of filariasis. If similar results are obtained in different filariasis-endemic countries, this method may be useful in global filariasis elimination programs.

INTRODUCTION

Numerous surveys conducted in the People’s Republic of China between 1956 and 1980 showed that lymphatic filariasis caused by Wuchereria bancrofti and Brugia malayi were endemic in as many as 864 counties in 16 provinces/autonomous regions/municipalities, with 25.6 million microfilaremic cases and 5.4 million clinical cases,1 which resulted in a huge personal, social, and economic burden.2 The Government of China has made enormous efforts to eliminate filariasis from the entire country, and has conducted one of the most successful anti-filariasis campaigns in the world. The infection disappeared in almost all filariasis-endemic areas by 2004, and the declaration of elimination of lymphatic filariasis is soon expected.3

In the final stage toward elimination, complete surveillance to verify the absence of filariasis transmission is necessary in the previously filariasis-endemic areas and among the floating population, in which night blood surveys to detect microfilariae have played an essential role in China. However, parasitologic methods are labor-intensive and not very sensitive. It is therefore desirable to have a complementary diagnostic method to support elimination efforts. Immunodiagnosis to detect filaria-specific antibodies will be useful in this situation. However, all immunologic methods used so far in control programs to diagnose lymphatic filarial infections required blood collection, which reduced compliance. This situation is particularly true when infection prevalence has reached a low level and when most people have become unfamiliar with the disease.

To overcome this problem, a new enzyme-linked immunosorbent assay (ELISA) that uses urine samples and detects filaria-specific IgG4 was developed.4 This ELISA showed a sensitivity of 95.6% with W. bancrofti-infected subjects in Sri Lanka, and a specificity of 99.0% with controls from areas in Laos, Thailand, and Japan not endemic for filariasis. When the urine ELISA was used in a filariasis-endemic village in Sri Lanka, the positive rate was 76.5%, which was 2.5 times higher than the rate obtained with the immunochromatographic card test (ICT). Among children 1–10 years of age (n = 68), the ELISA-positive rate (72.1%) was 2.1 times higher than the ICT-positive rate,5 which suggested that the urine ELISA could detect new infections (or filarial transmission) more effectively than the ICT. Because of its high specificity, the urine ELISA could also be used to confirm the elimination of filariasis in young children born after filariasis transmission had ended. These children should have negative results for filaria-specific antibodies in urine.

In China, there are two steps in confirming elimination of filariasis. The first is basic elimination at which the microfilaria (mf) rate in a filariasis-endemic county or city has been reduced to less than 1% through intervention. The second is the certification of elimination when the following three conditions are met: 1) basic elimination has been maintained for more than 10 years, 2) properly designed parasitologic surveys do not detect any mf-positive person, and 3) there are no vector mosquitoes infected with human filarial parasites.3 The first condition is based on the observation that mf-positive persons become negative without treatment within 10 years when the mf-positive rate in a community is reduced to a low level (< 1%).3 Thus, theoretically, children ≤10 years of age should not have filaria-specific antibodies when elimination of filariasis was certified. In the present study, urine ELISA was applied to school children in two different areas where the elimination had been certified some years before.

MATERIALS AND METHODS

Study area, subjects, and urine collection. Yongjia County (population = 742,209), Zhejiang province (Figure 1), was a W. bancrofti-endemic area with an overall mf prevalence of < 5% before control measures were taken. In 1956, mf-positive persons were treated with diethylcarbamazine

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(DEC) for the first time. Repeated blood surveys and treatments of known mf-positive persons with DEC were conducted in 1979–1985. This program was concluded by mass treatment with DEC-medicated salt in 1987. Basic elimination of filariasis was accomplished and elimination was certified in 1998. In September 2002, Yongjia Experimental Primary School was visited and students 6–10 years of age, as determined by school grade, were requested to provide urine samples. This school is located in Shangtang Township, which was one of the townships in Yongjia County with a high prevalence rate (5.1–20.0%) for filariasis before control measures were taken.

Gaoan city (population = 666,585) (Figure 1) had been endemic for bancroftian filariasis, with the average mf-positive rate of 1.4% in 1971–1982. Selective DEC treatments of mf-positive persons and three rounds of mass drug administration were carried out between 1983 and 1987. Blood surveys conducted in 1988–1990 showed an mf-positive rate of 0.77% and basic elimination had been achieved. During 1991–1998, the mf-positive rate remained low (< 1%). In 1999, DEC-medicated salt was given for two months in selected villages and the elimination of filariasis was certified in 2002. In December 2004, 57 schools (31 primary schools and 26 junior middle schools) (Figure 1) were visited and the students were requested to provide urine samples.

The study was reviewed and approved by the ethical committee of Aichi Medical University in Japan and the Chinese Center for Disease Control and Prevention. School children and their guardians and teachers were provided thorough explanations of the purposes and methods of the study and their consent was obtained. Each student was registered, asked about place of residence, and then requested to collect a urine sample in a plastic cup. Local health staff measured 5 mL of urine sample into a plastic bottle and then added sodium azide (0.1% final concentration). Samples were stored at 4°C until IgG4 was measured at Aichi Medical University School of Medicine, Japan. Such samples can be kept at least for four years with little deterioration in antibody titers (Itoh M and others, unpublished data).

**Urine ELISA and other tests.** Filaria-specific urinary IgG4 was measured by ELISA following the method we previously reported with slight modifications. Briefly, 96-well microtiter plates were coated with *B. pahangi* adult crude antigens (5 μg/mL), and blocked with casein buffer. Urine samples (100 μL/well) were applied without concentration and incubated overnight at 25°C. The plates were washed, peroxidase-conjugated monoclonal mouse anti-human IgG4 antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) was added, and the plates were incubated for one hour at 37°C. A color reaction was measured after the addition of 2, 2'-azino-di(3-ethylbenzothiazoline-6-sulfonate) peroxidase substrate. Diluted positive standard sera were prepared for each plate to generate a calibration curve from which an antibody unit was estimated. The unit in this system ranged from 0 to 7,290 U, and the cut-off value, which is the mean unit + 3 SD determined with samples from areas of Laos and Thailand not endemic for filariasis, was 54.7 U. In Yongjia, each urine sample was measured in duplicate, and average units were used. In Gaoan, only one sample was used.

Antibody-positive children were revisited six months after the first examination for new urine samples. There was no treatment given between the two examinations. In Gaoan, antibody-positive children were examined for urinary IgG4 and for microfilariae and filarial antigen with the Og4C3 ELISA. This ELISA used whole blood samples absorbed on filter paper following the method we previously reported. Family members of urinary IgG4-positive students were checked by the ICT.

**RESULTS**

**Yongjia study.** Urine samples were collected from 2,411 students 6–10 years of age. There were 1.7 times more boys than girls. Most (1,786) were from Shangtang Township and the remainder (625) were from 43 other townships in Yongjia County. Sixteen samples were judged positive in the first measurement. However, in four of them, opaque precipitation was noticed at the bottom of the well, which is apparently not the result of an immunologic reaction. Repeated measurements with such samples showed that the precipitation was not a constant phenomenon. Thus, all wells were checked for visible precipitation, and any urine that produced this precipitate was re-examined. These four samples were negative in the second or third examinations when there was no precipitation. There were also samples whose optical density (OD) values in duplicate were very different. For example, two samples had OD values of 0.016 and 0.839 (1.9 U and 1,074.1 U). It was then decided arbitrarily to re-examine a sample that was judged positive if the difference between two OD values was more than 40% of their average. There were 10 such samples, and all were negative when re-examined. Eventually, only 2 (0.08%) of 2,411 samples were positive for urinary IgG4. The distribution of antibody units is shown in Figure 2 according to age. The two positive samples had low titers (63 U each). They were re-examined six months later and showed negative results (19 U and 0 U).

**Gaoan study.** A total of 7,998 students 5–16 years of age were examined. Similar to Yongjia, there were 1.7 times more
boys than girls. There were 20 samples with opaque precipitation. All but one was negative when re-examined. One urine sample produced precipitation in repeated examinations. A total of 28 samples (0.35%) was positive (Figure 3). The 28 samples plus 1 sample that produced precipitation in all measurements were re-examined for urinary IgG4 in duplicate six months later; all were negative. For confirmation, a night blood survey for microfilaremia and Og4C3 ELISA for filarial antigen were also conducted during the visit. All results were negative. Moreover, samples from 25 fathers, 14 mothers, and 4 other family members of the 29 students were tested with ICT card tests; all samples were negative.

**DISCUSSION**

The urine ELISA has a definite advantage in terms of sample collection compared with blood-based diagnoses. The sensitivity and specificity of the ELISA were reported to be sufficiently high, 95.6% and 99.0%, respectively, but this ELISA needs to be re-assessed under different field conditions. Although IgG4 titers fluctuate during the day, urine samples from the same individuals showed positive results. In another study, the ELISA was used with samples from 203 children less than five years of age in a Sri Lankan village where the mf rate of all population was 5.7%. Four positive samples were found within 58 days of birth, which suggested possible transfer of IgG4 from mothers; and after 2 years of age, the IgG4 positive rate exceeded 24%. In an area of Thailand with low endemicity for filariasis, urinary IgG4 was detected in 2.7% of children ≤ 10 years of age (n = 75) and in 12.1% of persons 11–20 years of age (n = 116). These studies and the previously mentioned result in Sri Lanka suggest that the urine ELISA is effective in detecting filarial infection in young children and has a better sensitivity than mf and ICT tests. As for specificity, the urine ELISA had not been tested in a large-scale field study. When this ELISA was used with *B. pahangi* crude antigens and serum samples, false-positive reactions were reported in human Strongyloides infection (5 of 38 samples examined). Another study reported cross-reactions with samples from persons with onchocerciasis, loaasis, and echinococcosis, but not with samples from persons with strongyloidiasis (n = 22).

The present study of 10,409 students 5–16 years of age in two previously filariasis-endemic areas in China showed that urine ELISA positivity rates were low (0.08% in Yongjia County and 0.34% in Gaoan City). When compared with the 99.0% specificity obtained with samples from areas of Laos, Thailand, and Japan not endemic for filariasis (n = 298), these rates are much lower than expected. The present results endorsed the certification of elimination of filariasis and showed a high specificity of the urine ELISA. It is possible that prevalence and intensity of various parasitic infections in a community interfere with the ELISA by increasing background noise. In the case of Yongjia, intestinal parasitic infections seemed to be negligible at the time of this study. A report from Yongjia schools indicated that between 1989 and 1999, infection with *Ascaris* was reduced from 60.31% to 1.28%, infection with *Trichuris* from 54.77% to 3.22%, infection with hookworm from 9.31% to 0.00%, and infection with *Enterobius* from 26.14% to 5.82%.

All positive children were re-examined by testing new urine samples. Two positive samples from Yongjia with antibody values close to the cut-off value (63U for each) were negative in the second examination. In Gaoan, 28 positive subjects were examined for microfilariae, filarial antigen, and urinary IgG4. All subjects, including some with high amounts of urinary IgG4 (e.g., 4,174 U and 745 U in the first examination), were negative. Forty-three of their family members were also negative for *W. bancrofti* antigen. These findings suggest that a low IgG4 positivity rate among children is caused by false-positive reactions and that antigen tests are useful to confirm this finding.

In Yongjia, all samples were measured in duplicate. Any positive samples with a large difference between the two OD values (> 40% of the average) were re-examined; all were found to be negative. In Gaoan, antibody units were determined using only one well/sample. This is probably the reason why samples from Gaoan showed a higher prevalence in the first examination. In future studies, when one well/sample is used, re-checking of known positive samples in duplicate would be useful in excluding false-positive reactions. The cause of a large discrepancy in OD values using the same urine sample is probably technical but it is not known. Fortunately, such extreme differences were rare (< 0.42%). In the present study, some samples produced a precipitate at the
bottom of the well, although this observation was rare and observed only in 0.17% and 0.25% of the samples, respectively, in Yongjia and Gaoan. This finding had not been previously observed. Urine may contain a variety of unusual substances from food and medication, which might cause the precipitation. However, it is not known why the same sample did not always produce a precipitate.

It is relatively easy to monitor the reduction in prevalence and intensity of filarial infection. However, confirming this elimination is a more complicated and time-consuming process. In China, a minimum of 10 years was required after basic elimination had been achieved. Annual mass drug treatments with DEC (or ivermectin) and albendazole are being given in filariasis-endemic countries under the Global Program to Eliminate Lymphatic Filariasis. The treatment will be repeated for five years. To determine if additional mass treatments are necessary, the filariasis elimination program in each country must have reliable information on filariasis transmission. The ICT card tests for children are a suitable way to obtain this information. The present results with young children suggest that the urine ELISA could supplement the ICT-based survey or might be used instead if necessary. However, the ELISA must be validated in various filariasis-endemic regions with different epidemiologic features. The usefulness of the urine ELISA in areas endemic for *B. malayi* needs to be determined. The use of recombinant antigens for urine-based diagnoses will be beneficial in some filariasis-endemic areas. In this regard, we have tested *W. bancrofti*-derived recombinant *Wb*-SXP-1 in a urine ELISA and obtained promising results.

Received January 30, 2006. Accepted for publication February 6, 2007.

Financial support: This study was supported by Grants-in-Aid for Scientific Research (B) no. 14406005 and no. 17406011 from the Japan Society for the Promotion of Science.

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REFERENCES


