TABLE 2. Detailed comparison of microscopy, nested PCR, and LAMP for malaria parasite detection and species identification

Parasite(s) detected by each method (no. of samples)[a]		
Microscopy	Nested PCR	LAMP[b]
P. falciparum (12)[c]	*P. falciparum* (12)	*P. falciparum* (12)
P. falciparum + *P. vivax* (5)	*P. falciparum* + *P. vivax* (4), *P. vivax* (1)	*P. falciparum* + *P. vivax* (4), *P. vivax* (1)
P. vivax (34)	*P. vivax* (30), *P. vivax* (1), *P. vivax* (1), *P. ovale* (2)	*P. vivax* (30), *P. vivax* + *P. falciparum* (1), negative (1), *P. ovale* (2)
P. malariae (12)	*P. malariae* (8), *P. malariae* + *P. vivax* (1), *P. ovale* (1), *P. malariae* (1), negative (1)	*P. malariae* (8), *P. malariae* + *P. vivax* (1), *P. ovale* (1), *Plasmodium* spp.[d] (1), *P. malariae* (1)
P. ovale (5)	*P. ovale* (5)	*P. ovale* (5)
Negative (53)	Negative (50), *P. falciparum* (1), *P. vivax* (2)	Negative (50), *P. falciparum* (1), *P. vivax* (2)

[a] Results that were nonconcordant between nested PCR and LAMP are underlined.
[b] LAMP assays were run twice; in all cases the two experiments gave the same results.
[c] Each row provides the results obtained with identical DNA samples.
[d] Positive for the genus *Plasmodium* by LAMP but negative by all four species-specific LAMPs.

Clinical sensitivity and specificity: comparison of microscopy, nested PCR, and LAMP. The results of microscopy, nested PCR, and LAMP are given in Table 2. Among 121 patients in whom malaria was suspected, 68 (56.2%) were positive by microscopy (mean ± SD, 6,007 ± 5,761 parasites/μl; range, 210 to 24,164 parasites/μl). Of these, 12 patients (9.9%) were diagnosed with *P. falciparum* infection (812 to 24,164 parasites/μl), 34 (28.1%) with *P. vivax* infection (210 to 20,678 parasites/μl), 12 (9.9%) with *P. malariae* infection (294 to 5,253 parasites/μl), 5 (4.1%) with *P. ovale* infection (513 to 8,124 parasites/μl), and 5 (4.1%) with mixed *P. falciparum* and *P. vivax* infection (924 to 3,710 parasites/μl). The remaining 53 samples were negative. Microscopy was used as the reference standard for comparison with the other methods.

Nested PCR detected malaria parasites in 67 of 68 microscopically positive samples (sensitivity, 98.5%). LAMP with the genus-specific primer set also detected 67 of 68 samples positive by microscopy (sensitivity, 98.5%). Among the 53 samples that were negative by microscopy, both nested PCR and LAMP detected malaria parasites in 3 samples (specificity, 94.3%). The three samples positive by LAMP but negative by microscopy were reread by an expert microscopist; however, no parasite was detected in the slides. These samples were also retested by both nested PCR and LAMP. All three samples were again positive by LAMP and PCR; one was found to contain *P. falciparum* and the other two were found to contain *P. vivax*. Because of the agreement between PCR and LAMP, we believe that these three samples are true positives. Overall, both LAMP and nested PCR yielded results very similar to those of microscopy; the exceptions were 11 nonconcordant results (9.1%) each for nested PCR and for LAMP. These were mainly in the cases of *P. vivax*, *P. malariae*, *P. ovale*, or mixed infections. In contrast, only four samples (3.3%) had nonconcordant results between nested PCR and LAMP (Table 2).

LAMP yielded results in about 25.7 ± 4.9 min (mean ± SD; range, 19.4 to 52.9 min) for detection of the genus *Plasmodium* in the 67 samples that were positive by microscopy. The detection times were 31.7 ± 4.8 min (range, 25.8 to 44.9 min) for 17 *P. falciparum* samples, 30.6 ± 5.2 min (range, 25.4 to 46.6 min) for 37 *P. vivax* samples, 34.8 ± 4.8 min (range, 30.5 to 46.6 min) for 10 *P. malariae* samples, and 36.1 ± 6.8 min (range, 29.9 to 49.8 min) for 8 *P. ovale* samples. The average copy numbers of the target genes in the clinical samples were calculated on the basis of the linear regression formulas from the analysis of serial dilutions of plasmid DNA (Fig. 2B, D, F, H, and J). The average log copy numbers in clinical samples were 3.95 for the *Plasmodium* genus, 5.02 for *P. falciparum*, 3.78 for *P. vivax*, 4.10 for *P. malariae*, and 3.17 for *P. ovale*.

DISCUSSION

In regions where malaria is endemic, cases of mixed infections with malaria parasites are expected, so there is need for a test for species-specific identification that is more reliable than microscopy. Nested PCR is one such method; we have developed a simpler method for the diagnosis of clinical malaria diagnosis, including species identification, based on LAMP. The LAMP developed in this study has a sensitivity similar to that of microscopy and a specificity that is better than that of microscopy, and it yielded results similar to those of nested PCR for the detection of four species of human malaria parasites.

Since 96.7% of the results of LAMP were consistent with those of nested PCR and the sensitivity (98.5%) and specificity (94.3%) of both LAMP and nested PCR compared with the results of microscopy were equally high, we can propose that LAMP is as reliable as nested PCR for the clinical detection of four species of malaria parasites. Among five samples positive for both *P. falciparum* and *P. vivax* by microscopy, one was positive only for *P. vivax* by both nested PCR and LAMP. The level of *P. falciparum* parasitemia (approximately 0.01%) was lower than that of *P. vivax* parasitemia (approximately 0.05%) in this sample. Since the level of parasitemia was within the level detectable by nested PCR or LAMP, there may be another reason why this sample was negative for *P. falciparum* by both nested PCR and LAMP, such as the use of a smaller amount of blood or a lower efficiency of extraction of DNA from this specimen. Even though the LAMP results were in good agreement with those of nested PCR, there were some nonconcordant results. Among 12 samples positive for *P. malariae* by microscopy, 1 was negative by nested PCR and another 1 was negative by the species-specific LAMP. However, these two samples were successfully amplified by the *Plasmodium* genus-specific LAMP. One explanation is that the target sequences of the rRNA gene of *P. malariae* have variant sequences (9); thus, new primer sets for both nested PCR and

LAMP would be required to target regions that are universally conserved among *P. malariae* variants for reliable diagnosis. Among the samples found to contain *P. ovale* by both LAMP and PCR, microscopy had detected *P. ovale* in five of them, *P. vivax* in two samples, and *P. malariae* in the last sample. In contrast, both LAMP and nested PCR could successfully detect *P. ovale* in all eight samples, including both the LS type (six samples) and the CDC type (two samples), as confirmed by the *P. ovale* type-specific PCR method (28) (data not shown). On the basis of the comparable results between the LAMP and the nested PCR methods, as described above, the overall specificities of LAMP and nested PCR may be greater than that of microscopy.

The LAMP method can be used to quantify parasites in infected blood, although it is not a perfect assay for quantification. To quantify the parasites in infected blood by LAMP, we applied the average threshold time for the clinical blood samples to the formula of linear regression for each genus- and species-specific analysis. While there was a statistically significant linear correlation between the threshold time and the log of the initial copy number of template DNA, the linear correlation was rather poor for low copy numbers of template DNA (Fig. 2B, D, F, H, and J). Possible explanations are that (i) the longer incubation time before the detectable amplification caused by the lower template copy number makes the enzyme activity lower and (ii) the lower template DNA concentration, which decreases the annealing efficiency of the primers, may cause a delay in the threshold time. Although further improvement is needed, the statistically significant negative correlations between the threshold time and the parasite count by microscopy were obtained for the genus-specific LAMP-positive samples ($n = 67$), the samples positive for *P. vivax* only ($n = 30$), and the samples positive for *P. falciparum* only ($n = 12$) by using Spearman's rank correlation test (genus-specific LAMP, $P = 0.0002$; *P. vivax*-specific LAMP, $P < 0.0001$; *P. falciparum*-specific LAMP, $P = 0.0034$).

Diagnosis by LAMP does not require expensive reagents for DNA extraction, a turbidimeter, a thermal cycler, or skilled technicians. The template can be prepared by direct heat treatment of blood samples, without time-consuming and expensive DNA extraction with a commercial kit (21). Moreover, LAMP requires only a simple incubator, such as a heat block or a water bath that provides a constant temperature of 60°C, which makes it more economical and practical than nested PCR and real-time PCR. The white turbidity that results from magnesium pyrophosphate accumulation as a by-product of DNA amplification can be detected with the naked eye or a turbidimeter (13). Alternatively, inspection for amplification can be simply be performed with the naked eye by using SYBR green I, which turns green in the presence of amplified DNA. The results obtained by use of SYBR green I were consistent with those deduced by use of the real-time turbidimeter (18). Since the turbidity assay can be carried out in a closed system, the risk of contamination is lower than that when agarose gel electrophoresis is used. This is an additional advantage of LAMP for clinical use (2, 21, 23). Screening by genus-specific LAMP and then confirmation by a species-specific LAMP may provide a simple and reliable test for epidemiological surveillance. However, the routine screening for malaria parasites in both clinical laboratories and malaria clinics in areas where malaria is endemic may require a species-specific LAMP. Background epidemiological information will be helpful for local governments to decide how to apply LAMP for the diagnosis of malaria in each area.

In conclusion, the LAMP methods developed in this study can be useful for clinical diagnosis and active surveillance of malaria parasites in countries where malaria is endemic because it has a sensitivity and a specificity similar to those of nested PCR, requires minimal laboratory facilities, and is simpler and less expensive to perform than nested PCR. The on-site performance of LAMP at malaria clinics in the field will be required for further evaluation of this technique for the rapid diagnosis of malaria.

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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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FIGURE 1. Maps showing the study sites in Yongjia County and Gaoan City, People's Republic of China, and the distribution of 31 primary and 26 junior middle schools examined in Gaoan City. The divided areas in the city map represent administrative villages. ▲ = Primary school; ● = Junior middle school.

(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[4] 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.[6] 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) and 0.034 and 0.719 (30.7 U and 1,002.9 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

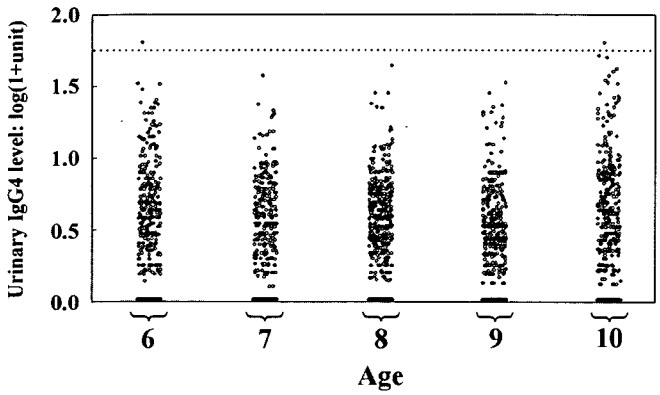

FIGURE 2. IgG4 antibody levels to *Brugia pahangi* in urine examined with 2,411 primary school students in Yongjia County, People's Republic of China. The dotted horizontal line indicates the cutoff value. Age is given in years.

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,[4] 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.[4] 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%.[7,8] 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%.[8] 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).[9] These studies and the previously mentioned result in Sri Lanka[5] 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).[10] Another study reported cross-reactions with samples from persons with onchocerciasis, loaiasis, and echinococcosis, but not with samples from persons with strongyloidiasis (n = 22).[11]

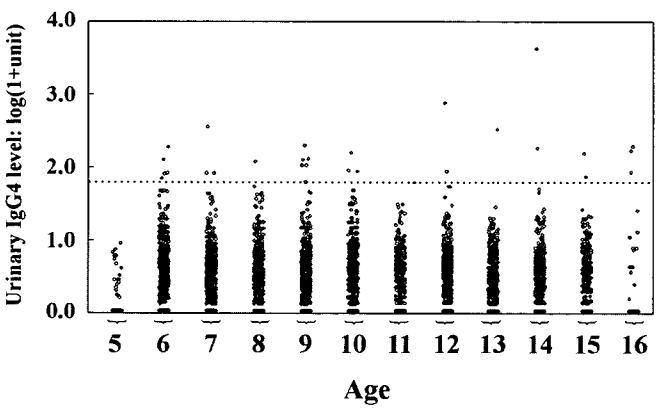

FIGURE 3. IgG4 antibody levels to *B. pahangi* in urine examined with 7,998 students at 57 schools in Gaoan City, People's Republic of China. The dotted horizontal line indicates the cutoff value. Age is given in years.

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 necessitated. 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[12] in a urine ELISA and obtained promising results.

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Multicentre evaluations of two new rapid IgG4 tests (WB rapid and panLF rapid) for detection of lymphatic filariasis

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Abstract

In the global effort to eliminate lymphatic filariasis (LF), rapid field-applicable tests are useful tools that will allow on-site testing to be performed in remote places and the results to be obtained rapidly. Exclusive reliance on the few existing tests may jeopardize the progress of the LF elimination program, thus the introduction of other rapid tests would be useful to address this issue. Two new rapid immunochromatographic IgG4 cassette tests have been produced, namely WB rapid and panLF rapid, for detection of bancroftian filariasis and all three species of lymphatic filaria respectively. WB rapid was developed using *Bm*SXP recombinant antigen, while PanLF rapid was developed using *Bm*R1 and *Bm*SXP recombinant antigens. A total of 165 WB rapid and 276 panLF rapid tests respectively were evaluated at USM and the rest were couriered to another university in Malaysia (98 WB rapid, 129 panLF rapid) and to universities in Indonesia (56 WB rapid, 62 panLF rapid), Japan (152 of each test) and India (18 of each test) where each of the tests underwent independent evaluations in a blinded manner. The average sensitivities of WB rapid and panLF rapid were found to be 97.6% (94%–100%) and 96.5% (94%–100%) respectively; while their average specificities were both 99.6% (99%–100%). Thus this study demonstrated that both the IgG4 rapid tests were highly sensitive and specific, and would be useful additional tests to facilitate the global drive to eliminate this disease.

Findings

Diagnostic tools are an essential component for the success of the Global Program for Elimination of Lymphatic Filariasis (GPELF). Thus far, the established diagnostic tests that are commercially available for bancroftian filariasis are two antigen detection tests namely NOW Filariasis Test [1] and Og4C3-ELISA (Trop Bio, Pty. Ltd., Australia); and for brugian filariasis is the Brugia Rapid test [2]. A laboratory-based Bm14-ELISA has also been extensively employed in studies in Egypt [3,4]. In addition

PCR-based assays for both brugian and bancroftian filariasis are also promising tools for the GPELF which can be employed for monitoring infections in both human and vector [5,6]. LF mainly affects the poor who reside in areas which are remote and/or without adequate health and laboratory facilities. Thus diagnostic tools in the format of rapid tests, particularly those based on immunochromatography technology, are most suitable to be employed for the GPELF, since they allow easy on-site testing, followed by rapid, simple reading and interpretation of results. These would avoid potential logistical challenges for sample storage and transportation, as well as more serious problems such as sample mix-up due to unclear/unreadable labels and sample degradation that may occur if collection and performance of tests are not conducted at the same or nearby locations. For such a major global program which needs to be sustained for a prolonged period, availability of a panel of rapid tests would help ensure smooth progress of the program and avoid potential problems such as supply interruption and changes/variations in test performance. Two new rapid immunochromatographic cassette tests based on detection of anti-filarial IgG4 antibody are now commercially available namely WB rapid and panLF rapid. The aim of this study is to perform a multicentre study to validate the sensitivities and specificities of the tests.

The test kits were acquired by the senior author from the manufacturer. A proportion of the tests were validated at USM, and the rest of the tests were couriered to the other four participating laboratories. The WB rapid test consists of two lines namely a test line and a control line, with the former comprising *Bm*SXP recombinant antigen.

The panLF rapid test consists of three lines namely two test lines, one comprising *Bm*SXP and the other *Bm*R1 recombinant antigens; and a control line. Goat anti-mouse IgG antibody is employed as the control line for both tests. These lines are invisible in an unused test and are coloured red after performance of the test. Serum/plasma and whole blood may be employed as test samples.

For serum samples, the test is performed by delivering 25 ul serum sample into the square bottom well. When the sample front reaches the blue line on the cassette window, two drops of buffer are added to a top oval well to release the conjugate solution (monoclonal anti-human IgG4 conjugated to colloidal gold). This is followed by pulling a plastic tab at the bottom of the cassette and adding a drop of buffer into the square bottom well, and by 15 minutes, the results can be read. For both tests, appearance of only the red control line denotes a negative result. For WB rapid test, a positive result is demonstrated when two red lines (a test and a control line) are seen. For panLF rapid test, a test is interpreted as positive when either three red lines (two test lines and a control line) or two red lines (a test and a control line) are observed.

Each participating institutions employed serum samples from their serum bank, which were obtained according to the ethical requirements of the respective organizations. With regard to the samples tested in Japan, the sera from *W. bancrofti* patients were collected in Sri Lanka, while the normal sera were from Japanese. The tests were performed in a blinded manner and the results were collected from each centre by e-mail attachments.

Table 1 shows the number of the tests and the results obtained at each institution. WB rapid test displayed an average sensitivity of 97.6% (239/245), ranging from 94% to 100%. The average overall sensitivity of panLF rapid test was 96.5% (390/404), ranging from 94% to 100%; the sensitivity for detection of *W. bancrofti* infection was 96.0% (217/226) [94% to 100%] while that for detection of brugian filariasis was 97.2% (173/178) [92% to 100%]. The specificities of both tests were evaluated with serum samples from quite a large variety of other infections, which included helminthes, protozoan, bacterial and viral infections. The results showed that the tests were either 99% or 100% specific, with an average specificity of 99.6%. Thus, at all the evaluation centres, the sensitivites and specificities of both tests were consistently high.

The mf+ samples with circulating filarial antigen (CFA), as determined by Og4C3 assay in samples from Sri Lanka (n = 41) and India (n = 18), had CFA values > 512 and >1000 respectively. The Sri Lankan mf- samples had CFA values > 512; with 62/63 (98.4%) and 59/63 (96.7%) samples positive for WB rapid and panLF rapid respectively. In addition, the two rapid tests were also tested with samples from 22 amicrofilaraemic, CFA+ individuals (cryptic infections) from India which had a wider range of CFA values (100–4786). In general both rapid tests tested positive with sera which had CFA units greater than 200, and they tested negative with sera which had CFA units below this value. Since CFA may remain positive for sometime after death of adult worms, some of the mf-, low CFA+ individuals may no longer be actively infected. On the other hand these may also be individuals with reproductively immature worms.

In the pre-certification phase of the elimination program and in the surveillance activities post-elimination, a highly sensitive test, as displayed by an antibody-based diagnostic tool is essential since the level of infection, if any, is very low. Therefore, although a rapid antigen detection test is already available for bancroftian filariasis, an antibody detection assay would probably be more useful

Table 1: Sensitivities and specificities of WB rapid and panLF rapid tests evaluated at five institutions.

	WB rapid					panLF rapid				
	Wb	Bm/Bt	*Other infec-tions	Nor-mals	Sens & Spec	Wb	Bm/Bt	*Other infections	Normals	Sens & Spec
UI	43 (43)	-	0 (13)	-	100% sens 100% spec	24 (24)	35 (38)	-	-	Overall sens: 95% Wb: 100% sens Bm: 92% sens
UM	30 (30)	-	0 (58)	0 (10)	100% sens 100% spec	30 (30)	29 (29)	0 (60)	0 (10)	100% sens (Bm & Wb) 100% spec
AMU	102 (104)	-	-	0 (48)	98% sens 100% spec	99 (104)	-	-	0 (48)	95% sens (Wb) 100% spec
USM	47 (50)	-	1 (65)	0 (50)	94% sens 99% spec	47 (50)	109 (111)	1 (65)	0 (50)	Overall sens: 97% Wb: 94% sens Bm: 98% sens 99% spec
ILS	17 (18)	-	-	-	94% sens	17 (18)	-	-	-	94% sens (Wb)

Note: Numbers indicate number of samples that were positive, and numbers in parenthesis were the number of samples tested.
UI : University of Indonesia, Indonesia
UM : University of Malaya, Malaysia
AMU : Aichii Medical University, Japan
USM : Universiti Sains Malaysia, Malaysia
ILS : Institute of Life Sciences, India
sens : sensitivity; spec : specificity
Wb: *Wuchereria bancrofti* ; Bm: *Brugia malayi* ; Bt: *Brugia timori*
Filarial samples tested in Indonesia, Malaysia and India came from mf+ individuals; while those tested in Japan also came from individuals who are mf-, CFA+ (>512 units).
*Other infections: ascariasis, trichuriasis, hookworm, strongyloidiasis, toxocariasis, toxoplasmosis, typhoid, cysticercosis, schistosomiasis, malaria, dengue, amoebiasis
WB rapid: sensitivity : 97.6% (239/245); specificity : 99.6% (243/244)
panLF rapid: overall sensitivity : 96.5% (390/404); sensitivity for Wb detection: 96.0% (217/226); sensitivity for Bm/Bt detection: 97.2% (173/178); specificity: 99.6% (232/233)

in the screening of young children as required in the pre-certification phase of GPELF. Antigen detection assays depend on presence of developmentally mature worms while antibody assays could potentially detect exposure to infective larvae by children. Thus WB rapid would be helpful to address this diagnostic requirement. For detection of all species of lymphatic filariasis, a rapid test such as panLF rapid would be very useful in several kinds of situations, namely testing in areas where there are mixed bancroftian and brugian filaria infections, in areas where the infecting species is not known or not confirmed, and for screening of immigrant workers in countries such as Malaysia which has more than 1.3 million workers from filarial endemic countries. These workers may pose a threat to the achievement of the disease elimination or they may be a source of resurgence of the disease in the future.

*Bm*R1 is a recombinant antigen derived from *Bm17DIII* gene [GenBank: AF225296] and employed in a rapid test called Brugia Rapid. It has been shown to be highly sensitive (>95%) and specific (≥ 99%) for detection of *B. malayi* and *B. timori* infections in laboratory evaluations [7-10] and field studies [11-14]. In a field study in Malaysia which is a low endemic area, Brugia Rapid detected about ten times more positive cases than parasitological diagnosis, while in the high endemic area of Indonesia, the increase in detection was about three times [11,12]. Follow-up post-treatment studies of mirofilaraemic individuals showed that the titres of IgG4 antibodies to *Bm*R1 decreased post-treatment. In Malaysia which is a low endemic area, it took approximately 6 months to 2 years post-treatment for the assay to become negative [15,16]. In a study involving a paediatric population in Kerala, ultrasonography ('filarial dance sign' or FDS) identified adult worms in 7 out of the 39 (18%) amicrofilaraemic children who were Brugia Rapid positive, thus providing definitive evidence that the rapid test detected active infection. This was comparable to the observation of FDS in 6 out of 32 (19%) microfilaraemic children [17].

*Bm*SXP is a recombinant antigen derived from *SXP1* gene [GenBank no: M98813], the clone was isolated from a *B. malayi* adult male worm cDNA library with sera of bancroftian filariasis patients [18]. A rapid flow-through IgG immunofiltration test using *Wb*SXP recombinant antigen has been developed and a sensitivity of 91% (30/33) was recorded for detection of *W. bancrofti* infection [9].

In a recent study, *Bm*SXP was found to be more sensitive (95%) in detecting *W. bancrofti* infection as compared to *Bm*R1 (14%). On the other hand *Bm*R1 was more sensitive than *Bm*SXP in detecting *B. malayi* infection (98% and 84% respectively) [19]. Since *Bm*R1 and *Bm*SXP recombinant antigen cross-reacts with bancroftian and brugian filaria infection sera respectively, the panLF rapid test is not useful for species identification. However in the context of GPELF or for screening of foreign workers, this does not pose a problem. Cross-reactivities with *Loa-loa* and *Onchocerca* infection sera were observed with both rapid tests, thus they are not useful in areas co-endemic with these infections. However the tests may be employed in

the vast lymphatic filariasis endemic areas in the world, particularly in Asia, which do not overlap with endemic areas for non-lymphatic filariasis. Since LF endemic areas are also prevalent for infections with soil-transmitted helminthes and intestinal protozoa, the high specificities shown by both rapid tests with respect to non-filarial infections would allow the tests to be used with high confidence in these areas.

In conclusion, the present multicentre evaluation study conducted in five institutions (located in four different countries) clearly demonstrated the high sensitivities and specificities of WB rapid and panLF rapid tests. Thus these tests should be employed in further field studies and would merit consideration as potential tools to assist in the GPELF.

Competing interests

RN, with the assistance of RAR, developed WB rapid and panLF rapid tests

Authors' contributions

RN – conceive, design and supervise the study, participated in the evaluation at USM, analysed the results, wrote the first draft of the manuscript.

RAR – performed the evaluation and participated in the analysis of the results at USM.

IM & KE-supervised and participated in the evaluation at Aichi Medical University, edited the manuscript.

RB – supervised and participated in the evaluation at the Institute of Life Sciences, edited the manuscript.

RM – supervised and participated in the evaluation at University of Malaya, edited the manuscript.

ST – participated in the evaluation at University of Indonesia, edited the manuscript.

WMV – supervised and participated in the serum sample collection in Sri Lanka, edited the manuscript.

All authors read and approved the final manuscript

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SHORT REPORT: PRODUCTION OF RECOMBINANT KINESIN-RELATED PROTEIN OF *LEISHMANIA DONOVANI* AND ITS APPLICATION IN THE SERODIAGNOSIS OF VISCERAL LEISHMANIASIS

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Abstract. To detect IgG antibody in the serodiagnosis of visceral leishmaniasis (VL), a recombinant antigen rK39, which is part of a *Leishmania chagasi* kinesin-related protein, has been used successfully and showed high sensitivity and specificity. We report production of a recombinant protein rKRP42, which is part of an *L. donovani* kinesin-related protein and a homolog of rK39, and its application in an enzyme-linked immunosorbent assay (ELISA) for the diagnosis of VL. When rKRP42 and rK39 were compared, amino acid sequence analysis showed 89.3% identity and 98.7% homology, with rKRP42 having 39 more amino acids than rK39. The ELISA using rKRP42 showed a sensitivity of 94.6% (70 positive samples among 74 from VL patients) and a specificity of 99.3% (148 negative samples among 149 samples from Japanese controls), whereas the sensitivity of the commercial rK39 dipstick test was 93.2% (69 positive samples among 74 from patients with VL). The rKRP42 is a promising new antigen in developing immunodiagnostic methods for VL.

Visceral leishmaniasis (VL) or kala-azar is caused by an intracellular protozoan parasite of the *Leishmania donovani* complex and is considered as one of the most neglected diseases.[1] More than 47 countries are currently affected, with at least 200 million people at risk.[2] Approximately 90% of the 500,000 estimated annual cases of VL occur in rural areas of Bangladesh, India, Nepal, Sudan, and Brazil in some of the world's poorest regions. This disease accounts for 75,000 deaths annually.[3]

Most VL cases in peripheral health facilities are still treated on the basis of clinical suspicion and/or the result of an inadequately sensitive and specific formol-gel test (aldehyde test).[4] However, classic clinical features of VL are shared by several other endemic diseases such as malaria, disseminated tuberculosis, and enteric fever, which are also common in many of the areas endemic for VL. Demonstration of the causative parasites in aspirates from lymph nodes, bone marrow, and spleen is the most specific diagnosis, with the sensitivities of 56.3%, 67.1%, and 93.3%, respectively.[5] These techniques are invasive and require skilled personnel and equipped facilities. Because of the high mortality if left untreated and the serious toxicity of the most widely used first-line drug, sodium stibogluconate, a highly sensitive and specific diagnostic method that is simple, inexpensive, and applicable in rural settings is urgently needed.

Several serologic tests, such as enzyme-linked immunosorbent assays (ELISAs) with crude or recombinant antigens[6–8] and the direct agglutinin test (DAT),[9,10] have provided useful diagnostic results. Recently, a recombinant antigen rK39, which is part of an *L. chagasi* kinesin-related protein, has been widely evaluated by ELISA or in a dipstick format.[11,12] Although the antigen has been reported satisfactory, results varied considerably in different disease-endemic areas. Thus, it was desirable to develop new antigens for comparison. We report the production of recombinant protein rKRP42, which is part of an *L. donovani* kinesin-related protein and a homolog of rK39 and evaluation of this antigen in an ELISA with serum samples for the diagnosis of VL.

Leishmania donovani strain DD8, isolated from a Bangladeshi patient, was used.[13] Promastigotes were cultured and harvested as described previously.[14] Genomic DNA was extracted from promastigotes by phenol extraction method. To obtain the gene coding the rK39 homolog, polymerase chain reaction (PCR) amplification was performed by using primers (rK39 sense, 5′-GAGCTCGCAACCGAGTGGGAGGAC-3′ and rK39 antisense, 5′-CTGGCTCGCCAGCTCCGCG-GCGCG-3′) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and *L. donovani* genomic DNA. The amplified PCR product was subjected to electrophoresis on an agarose gel, purified with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), and ligated into the *Eco*RV site of the pBluescript KS(-) vector (Stratagene). The PCR product was confirmed to have 1,011 basepairs (GenBank accession no. AB256033). Further PCR amplification was performed with the sequenced-confirmed plasmid containing the rK39 homolog gene, known as rKRP42, as a template by using primers (rK39 sense, 5′-GAGCTCGCAACCGAGTGG-GAGGAC-3′ and 6His/*Eco*RI-rK39 antisense, 5′-GTGAT-GGTGATGGTGATGGAATTGATCCTGGCTCGC-CAGCTC-3′) with *Pfu* DNA polymerase. The PCR product coding rKRP42 and a six-histidine amino acid (6His) tag was cloned into the in *Sma* I site of the pTYB12 expression vector (New England Biolabs, Beverly, MA). The rKRP42 was purified with QIAexpress kit (Qiagen) and IMPACT™-CN System (New England Biolabs) according to the manufacturer's protocols.

Briefly, *Escherichia coli* cells were cultured with Luria-Bertani medium containing 100 μg/mL of ampicillin. Protein expression was induced with isopropyl thio-β-galactoside at a final concentration of 0.4 mM at 16°C for 16 hours. The cells were then harvested and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 0.1% Triton X-100). After sonication and centrifugation,

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the clarified cell extract was purified on an Ni-NTA column (Qiagen). The column was washed with the Ni-NTA wash buffer, and the extraction fraction was eluted with Ni-NTA elution buffer. The eluate was then purified on a chitin column (New England Biolabs) and washed with chitin column wash buffer. The column was kept with cleavage buffer containing dithiothreitol (DTT) at 16°C for 16 hours, and then rKRP42 was eluted with cleavage buffer without DTT (Figure 1). The rKRP42 antigen contains 337 amino acids and is one repeat (39 amino acids) longer than the rK39 antigen (Figure 2). The amino acid sequence of rKRP42 showed 89.3% identity and 98.7% homology with rK39 antigen.

We performed an ELISA with rKRP42 antigen as follows. Flat-bottomed 96-well microtiter plates (MaxiSorp™; Nunc, Roskilde, Denmark) were coated with 1 μg/mL (100 μL/well) of rKRP42 antigen and incubated overnight at 4°C. After blocking with casein buffer (1% casein in 0.05 M Tris-HCl buffer, 0.15 M NaCl, pH 7.6) for two hours at room temperature, 100 μL of serum (1:4,000 dilution in casein buffer) was added to the wells and incubated for one hour at 37°C. After four washes with phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20, peroxidase-conjugated goat anti-human IgG (Tago, Camarillo, CA), diluted 1:4,000 with casein buffer was added and incubated for one hour at 37°C. After four washes, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, MD) was added and incubated for one hour at room temperature. The optical density was measured at 415 nm and at 492 nm as reference. Each sample was assayed in duplicate. If the absorbance values of the duplicate samples differed by > 40% from their average, the sample was retested. Antibody levels were expressed arbitrarily as units, which were was estimated from a standard curve constructed with serially diluted positive sera. The cutoff point for IgG to rKRP42 was calculated as the mean plus three standard deviations of log (unit + 1) values of the non-endemic healthy controls (NEHCs). The anti-logarithmic value was 109.4 units.

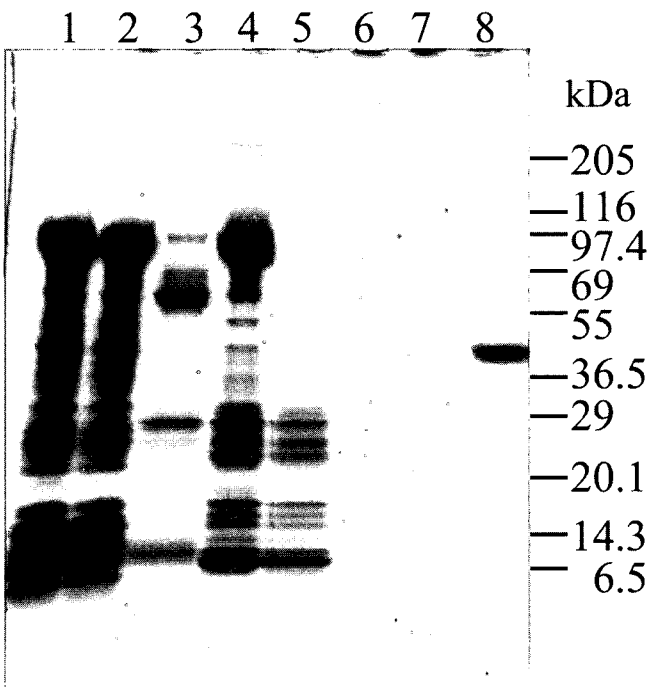

FIGURE 1. Purification of rKRP42 from induced *Escherichia coli* clone cells by using Ni-NTA and chitin columns. Protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and visualized by staining with Coomassie brilliant blue. Lane 1, Crude extraction from induced cells; lane 2, Ni-NTA column flow through fraction; lane 3, Ni-NTA column wash fraction; lane 4, Ni-NTA column elution fraction with the elution buffer; lane 5, chitin column flow through fraction; lane 6, chitin column wash fraction; lane 7, chitin column rinse fraction with the cleavage buffer; lane 8, chitin column elution fraction after 16-hour cleavage reaction. kDa = kilodaltons.

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rKRP42   ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESASERLTSLEQQLRESEERAVE  60
rK39     ELATEWEDALRERALAERDEAAAAELDAAASTSQNARESACERLTSLEQQLRESEERAAE  60

rKRP42   LASQLESTTAAKMSAEQDRENTRAALEQQLRESEERAAELASQLEATAAAKSSAEQDREN 120
rK39     LASQLEATAAAKSSAEQDRENTRATLEQQLRESEARAAELASQLEATAAAKMSAEQDREN 120

rKRP42   TRAALEQRLRESEARAAELASQLEATAAAKSSAEQDRENTRAALEQRLRESEERAAELAS 180
rK39     TRATLEQQLRDSEERAAELASQLESTTAAKMSAEQDRESTRATLEQQLRDSEERAAELAS 180

rKRP42   QLEATAAAKSSAEQDRENTRPALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRA 240
rK39     QLESTTAAKMSAEQDRESTRATLEQQLRESEERAAELASQLESTTAAKMSAEQDRESTRA 240

rKRP42   ALEQQLRESEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRESEERAAELASQLE 300
rK39     TLEQQLRDSEERAAELASQLEATAAAKSSAEQDRENTRAALEQQLRDSEERAAELASQ   298

rKRP42   ATAAAKSSAEQDRENTRAALEQRLRESEARGAELASQ                         337
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FIGURE 2. Comparison of amino acid sequences of rKRP42 and rK39 antigens in a single-letter code. Asterisks indicate identical residues and periods indicate conservative amino acid substitutions.

The rK39 antigen-based dipstick test (InBios International, Seattle, WA) was carried out according to the manufacturer's instruction. Briefly, 20 μL of serum was added to a test strip. The strip was placed in a well of 96-well microtiter plate, and two drops of chase buffer solution were added to each well. The test result was read within 10 minutes after addition of serum. Even a weak line was considered positive.

Seventy-four serum samples from defined VL patients collected from different medical college hospitals in Bangladesh were used to compute sensitivities of the rKRP42 ELISA and rk39 dipstick test. Among the 74 patients, 32 were confirmed parasitologically: Leishman-Donovan bodies were detected in smears of splenic aspirates (18 patients) or bone marrow aspirates (6 patients), and promastigotes were detected in 8 patients after inoculation of aspirate materials into Novy, MacNeal, and Nicolle medium. Of the other 42 clinically confirmed patients, 27, 7, and 8 were positive by conventional DAT, aldehyde test, and rK39 dipstick test, respectively. At the time of sample collection, all patients were being treated with sodium antimony gluconate at the recommended dose of the World Health Organization.[15] Sera containing preservative (NaN_3) at a concentration of 0.1% (w/v) were transported to Japan at ambient temperature and then stored at −40°C. Seventy-two samples from healthy Japanese individuals were used as NEHCs to determine the cutoff value. Another 149 NEHC samples were used to determine the specificity of the rKRP42 ELISA.

The study was reviewed and approved by the Ethics Committee of Aichi Medical University School of Medicine, Japan, and the Ethical Review Committee of the Bangladesh Medical Research Council.

The ELISA with rKRP42 antigen showed a sensitivity of 94.6% (70 positive samples among 74 VL samples) and a specificity of 99.3% (148 negative samples among 149 Japanese controls) (Figure 3). The sensitivity of the rK39 dipstick was 93.2% (69 positive samples among 74 VL samples). Because of the high specificities already reported for the rK39 dipstick test (97–100%),[11,16–19] Japanese controls were not tested. There are three parasitologically confirmed cases who were negative by both the ELISA and dipstick test. In a separate study, these three cases showed negative results with a conventional serum-based DAT, a urine-based ELISA with soluble antigen of acetone-treated promastigotes,[14] and a urine-based DAT.[20] One ELISA-positive, dipstick-negative sample had a relatively low antibody titer of 202.0 units. We could not determine the specificity for other diseases such as malaria, tuberculosis, and cutaneous leishmaniasis and for healthy controls from a disease-endemic area because of a lack of serum samples.

A variety of immunologic methods have been used to diagnose VL. Among others, the rK39 dipstick test is used because of its ease in handling, quick results, and high sensitivity and specificity. However, the sensitivity varied considerably in different disease-endemic areas. In India and Nepal,[16–18] the test showed the highest sensitivity (100%), but the sensitivity was significantly lower in Venezuela (88%),[21] southern Europe (71.4%),[19] and Sudan (67%).[22] This variation may be due to differences in the test accuracy between subspecies of *L. donovani* complexes, genetic differences in individual patients or in racial subgroups, and epidemiologic factors such as length or severity of diseases.[23] It would be worthwhile to test the new rKRP42 antigen in different geographic areas.

Some persons with VL do not show any clinical manifestations. Khalil and others[24] reported that in eastern Sudan the ratios of clinical and subclinical cases in 1994–1995 and 1995–1996 in Um-Salala village were 1.2:1 and 2.6:1, respectively, and in Mashrau Koka village were 1:11 and 1:2.5, respectively. In another study conducted in Bihar State, India, 69% of asymptomatic seropositve cases detected by the rK39 ELISA and dipstick test developed kala-azar within one year,[25] which suggested that that many of the asymptomatic cases were in a pre-clinical state. In predicting possible clinical cases, an ELISA that is quantitative would be more advantageous than a dipstick format; high antibody titers or an increase in antibody titers with time could be indicative of possible clinical cases. Such early diagnosis will have a practical importance now that oral treatment with miltefosine has become available.[26] Measurements of *Leishmania*-specific IgG, IgM, IgE, and IgG subclasses were also found to be useful as markers for active VL cases and for monitoring effective treatment.[27,28] The rKRP42 ELISA for IgG antibody can be used in clinical follow-up studies based on antibody titers and modified for various immunoglobulin classes.

Recently, the use of urine for blood has been considered valuable because of its ease in sample collection, and urine-based tests for the diagnosis of VL and other parasitic diseases have been reported.[14,20,29,30] Before establishing a urine-based immunodiagnostic method with a new antigen, the antigen must be first evaluated with serum samples. In a field study, when many borderline positive results can be expected, the serum-based rKRP42 ELISA can be a valuable reference.

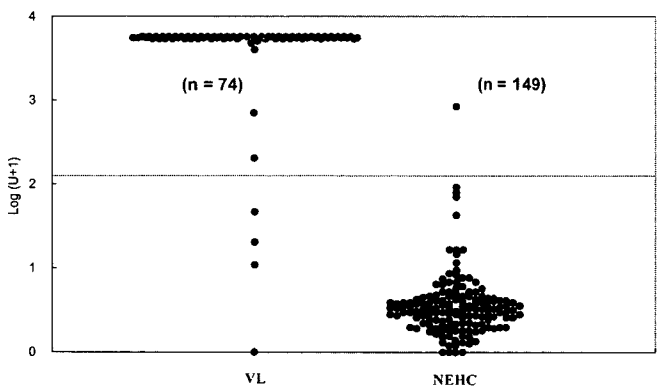

FIGURE 3. Detection of IgG antibody to rKRP42 in sera of patients with visceral leishmaniasis patients (VL) and non-endemic healthy Japanese controls (NEHC) by enzyme-linked immunosorbent assay. Each dot represents one serum sample. Thirty-three samples exceeded the highest measurable point (5,698 units) of the standard curve and were considered 5,698 units. The horizontal dotted line represents the cutoff value of 109.4 units.

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The ELISA-based detection of anti-*Opisthorchis viverrini* IgG and IgG_4 in samples of human urine and serum from an endemic area of north-eastern Thailand

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The levels of correlation between the number of *Opisthorchis viverrini* eggs excreted in the faeces and levels of anti-*Opisthorchis* IgG and IgG_4 in the serum and urine (as indicated by absorbances in ELISA) have recently been evaluated in north–eastern Thailand. The 225 subjects investigated in detail, all of whom came from an endemic village in Chaiyaphum province, were selected on the basis of the numbers of *O. viverrini* eggs that they were excreting. ELISA based on a crude antigen extract of the trematode were then used to determine the levels of specific IgG and IgG_4 in serum and urine samples. Compared with the egg-negative, the villagers who were found to be egg-positive for *O. viverrini* had significantly higher levels of specific IgG in their urine and serum and significantly higher levels of specific IgG_4 in their serum. The serum levels of specific IgG and IgG_4 and the urine levels of specific IgG all correlated with the numbers of *O. viverrini* eggs/g faeces [with correlation coefficients (r) of 0.251, 0.121 and 0.142, respectively]. Although the serum levels of IgG were positively correlated with the urine levels of IgG (r=0.098), there was no significant relationship between the serum and urine levels of specific IgG_4 (r=0.051).

When the 225 subjects investigated in the ELISA were divided according to whether they had no detectable *Opisthorchis* eggs in their faeces (N=57), or 1–100 (N=154), 101–1000 (N=5), 1001–1500 (N=5) or >1501 (N=4) eggs/g faeces, the serum and urine levels of specific IgG and the serum (but not urine) levels of specific IgG_4 were also found to correlate significantly with the infection-intensity categories (with r-values of 0.550, 0.146 and 0.578, respectively). When the results of the faecal examinations were treated as the 'gold standard', the ELISA for the detection of (*Opisthorchis*-specific) serum IgG, serum IgG_4, urine IgG and urine IgG_4 had sensitivities of 99.2%, 23.1%, 43.0% and 45.9% and specificities of 93.0%, 29.6%, 45.9% and 67.2%, respectively. Although the study was limited by the small number of subjects with intense infections, it appears worth investigating urine samples for subclasses of specific IgG other than IgG_4.

Human infection with the trematode *Opisthorchis viverrini* is endemic in South-east Asia, particularly Thailand, Laos, Cambodia and southern Vietnam (Tesana, 2005; Le *et al.*, 2006). Most people acquire the infection by eating raw, fermented or improperly cooked freshwater cyprinoid fish, in traditionally prepared meals (Tesana *et al.*, 1986; WHO, 1995). Those infected develop chronic hepato–biliary and gall-bladder diseases, often with fatigue and pain in the right-upper quadrant of the abdomen (Upatham *et al.*, 1982, 1984). The fluke is significantly linked with cholangiocarcinoma, and the incidence of this cancer reaches its highest level in north–eastern Thailand, where *O. viverrini* is

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particularly common (Vatanasapt *et al.*, 1990; Sriamporn *et al.*, 2004).

The routine diagnosis of human opisthorchiasis is based on the checking of faecal samples — as simple smears, Kato thick smears, or by formalin–ethyl-acetate concentration — for the trematode's eggs. Although, in the 1990s, Kato thick smears were used for a national survey of intestinal parasitic infection in Thailand (because they are relatively easy to prepare in the field and reasonably sensitive), the eggs of *O. viverrini* in the smears could not be differentiated from those of some 'minute intestinal flukes' (Jongsuksuntigul *et al.*, 1992). Using Kato smears, 24.1% of the subjects from north–eastern Thailand and 22.8% of those from the north were found positive for *O. viverrini* and/or 'minute intestinal flukes' (Jongsuksuntigul *et al.*, 1992). By using a combination of praziquantel treatment and microscopical examination of the adult trematodes excreted post-treatment, Radomyos *et al.* (1994, 1998) were able to show, however, that most (92.2%) of those infected with *O. viverrini* and/or 'minute intestinal flukes' in north–eastern Thailand but only a small percentage (11.6%) of apparently similar subjects from northern Thailand were actually infected with *O. viverrini.*

In the present study, as part of an attempt to develop a more sensitive and specific technique for the routine diagnosis of opisthorchiasis, ELISA for the detection of anti-*Opisthorchis* antibodies in urine and serum samples have been evaluated.

SUBJECTS AND METHODS

Collection of Serum and Urine Samples

Ban Nong Khon Thai, the village selected for the study, lies in Chaiyaphum province, close to the Chi River, in north–eastern Thailand. The villagers were informed about the methods and aims of the research project before written consent was obtained from each subject who met the inclusion criteria. Subjects aged <10 years of age, the pregnant, those with viral hepatitis, and those with other serious health problems were excluded.

The enrolled subjects were asked whether and, if appropriate, when they had taken praziquantel (or any other anti-parasitic drug) and eaten raw, fermented or under-cooked fish. A faecal sample from each subject was tested for the eggs of *O. viverrini* and for other intestinal parasites, using quantitative formalin–ethyl-acetate concentration. The counts of *O. viverrini* eggs/g faeces (which were used as an indicator of the intensity of *O. viverrini* infection) were used to select a subset of subjects, covering all observed intensities of *O. viverrini* infection. A urine sample and a 5-ml blood sample of blood was collected from each member of this subset. Each urine sample was mixed with sodium azide, to give a final concentration of 0.1% (w/v) sodium azide, and each blood sample was allowed to clot so that the serum could be separated off and stored at −70°C until it could be tested in the ELISA (see below).

Preparation of Crude Antigen

The ELISA were based on a crude extract of the somatic antigens of whole adult *O. viverrini.* The adult flukes were recovered from Syrian golden hamsters that had been infected, with metacercariae, 2 months earlier. After three washes with cold phosphate buffer, the flukes were homogenized in a mixture of 1 mM phenylmethyisulfonyl fluoride (PMSF; 10 µl), 0.1 mM *N*-tosyl-L-phenylalaninechloromethylketone (TPCK; 10 µl) and 2 mM mercapto-ethanol (100 µl), in an ice-bath. The homogenate was sonicated to give the aqueous suspension that was used as the antigenic extract. This extract was stored at 4°C until needed.

ELISA

The concentration of protein in the crude antigenic extract was determined, using the Lowry method (Lowry *et al.*, 1951), before

the extract was diluted with coating buffer, to give 10 μg protein/ml, and distributed, at 100 μl/well, in ELISA plates. After a 1-h incubation at 37°C, the plates were blocked with 5% skimmed milk and washed three times with phosphate buffer before a test serum diluted 1:400 or an undiluted urine sample was added to each well (at 100 μl/well, with the same volume of 2% skimmed milk). After a further incubation at 37°C, for 1 h (sera) or 24 h (urine samples), the plates were washed again before 100 μl of a conjugate of horseradish peroxidase with either goat anti-human IgG or mouse monoclonal anti-human IgG_4, diluted 1:10,000 in 2% skimmed milk, were added to each well. After a further incubation for 1 h at 37°C, the plates were washed and then an OPD (o-phenyldiamine and urea hydrogen peroxide) substrate was added for 15 min. The reaction was stopped with 2 M H_2SO_4 before absorbance at 492 nm was measured. Serum and urine levels of specific IgG and IgG_4 were recorded as absorbances. Any sample that gave an absorbance that was higher than the corresponding mean absorbance plus three S.D. for the relevant negative-control samples (of urine samples and sera from 12 subjects living in an area of central Thailand where *O. viverrini* is unknown) was considered positive for *O. viverrini*.

In estimates of the sensitivity and specificity of each ELISA, the results of the faecal examinations, excluding the data for the villagers who were found to have mixed infections, were used as the 'gold standard'.

RESULTS

Faecal samples were collected from 531 villagers, all living within 10 km of the centre of the study village. The faecal samples from 273 (51.4%) of the villagers were each found positive for at least one intestinal parasite. As the Table indicates, *O. viverrini* appeared to be the most common parasite, followed by minute intestinal flukes (29 infections), hookworm (20) and *Strongyloides stercoralis* (14). Thirty-two of the villagers were found egg-negative for *O. viverrini* despite having no history of anthelminthic ingestion. All of the subjects reported that they regularly ate meals that included raw, fermented and/or 'undercooked' freshwater fish.

ELISA

The 225 villagers investigated in the ELISA were selected to give a spread of intensities of *O. viverrini* infection, and included 57 egg-negatives as well as egg-positives who appeared to have very low (1–100 eggs/g; $N=154$), low (101–1000 eggs/g; $N=5$), moderate (1001–1500 eggs/g; $N=5$) or high (>1501 eggs/g; $N=4$) intensities of *O. viverrini* infection.

TABLE. *The parasitic infections detected, by faecal concentration, in 531 villagers from an area of Chaiyaphum province where* Opisthorchis viverrini *is endemic*

Parasitic infection	No. and (%) of villagers:		
	Infected	As single infection	As mixed infection
Opisthorchis viverrini	254 (47.8)	205	49
Hookworm	20 (3.8)	6	14
Taenia sp.	2 (0.4)	1	1
Minute intestinal flukes	29 (5.5)	1	28
Giardia lamblia	4 (0.8)	2	2
Strongyloides stercoralis	14 (2.6)	5	9
Sarcocystis sp.	1 (0.2)	1	0
None found	258 (48.6)		

Serum and urine levels of specific (i.e. anti-*Opisthorchis*) IgG and serum (but not urine) levels of specific IgG_4 were each significantly higher in the egg-positives than in the egg-negatives ($P<0.05$ for each). The serum levels of specific IgG and specific IgG_4 and the urine levels of specific IgG in the urine were all significantly and positively correlated with the number of *O. viverrini* eggs excreted/g faeces, with correlation coefficients (r) of 0.251, 0.121 and 0.142, respectively. Although the serum level of specific IgG was also positively correlated with the urine level of specific IgG ($r=0.098$), there was no significant relationship between the serum and urine levels of specific IgG_4 ($r=0.051$). Given these results, it was no surprise to find that the serum and urine levels of specific IgG and the serum levels of specific IgG4 also correlated with infection-intensity category (zero, very low, low, moderate and high), giving r-values of 0.550, 0.146 and 0.578, respectively. No corresponding correlation was found for urine levels of IgG_4, perhaps because the number of high-intensity infections was too small.

SEROPOSITIVITY

In terms of their serum levels of specific IgG (for which the threshold absorbance for positivity was 0.16), all but one of the subjects found egg-positive were also found seropositive, the exception being a villager excreting <100 eggs/g. This gave a high sensitivity, of 99.2%, but the number of seropositive egg-negatives observed (all of whom had been found infected with *Taenia*, minute intestinal flukes, *St. stercoralis*, hookworm or *Sarcocystis*) gave a specificity of just 23.1% [Fig. 1(a)].

Most of the egg-positive subjects were also found to be positive in terms of their serum levels of specific IgG_4 (exceeding the threshold absorbance of 0.01) but again, because many of the villagers who were egg-negative but found infected with other parasites were also found positive in terms of their serum levels of anti-*O. viverrini* IgG_4 [Fig. 1(b)], the ELISA for this subclass of IgG in serum appeared to have a poor specificity (29.6%) despite its high sensitivity (93.0%).

URINE POSITIVITY

In terms of their urine levels of specific IgG (for which the threshold absorbance for positivity was 0.11), many of the egg-positive villagers were also ELISA-positive [Fig. 2(a)]. There were, however, considerable numbers of egg-positive but ELISA-negative villagers, giving a relatively poor sensitivity of 43.0%, although few of the egg-negative villagers were found ELISA-positive (giving a specificity of 64.45%).

Only about half of the egg-positive subjects were ELISA-positive in terms of their urine levels of specific IgG_4 (for which the threshold absorbance for positivity was 0.19), giving a sensitivity of only 45.9% [Fig. 2(b)]. With the urine-IgG_4 ELISA, however, there was no apparent reactivity with parasites other than *O. viverrini*, and only one egg-negative but ELISA-positive villager was detected, giving a relatively high specificity of 67.2%.

DISCUSSION

Faecal concentration revealed a high prevalence of parasitic infection among the inhabitants of the study village in north-eastern Thailand (51.4%), with *O. viverrini* predominant (47.8%). Most of the mixed infections observed were of a food-borne parasite (*O. viverrini* or a 'minute intestinal fluke') with a soil-transmitted helminth (hookworm or *St. stercoralis*). Most of the villagers presumably put themselves at high risk of infection with *O. viverrini* by regularly consuming raw, fermented or improperly cooked freshwater fish. Given the high prevalence of *O. viverrini* infection, it was surprising that so few participants had intense infections with this parasite. The

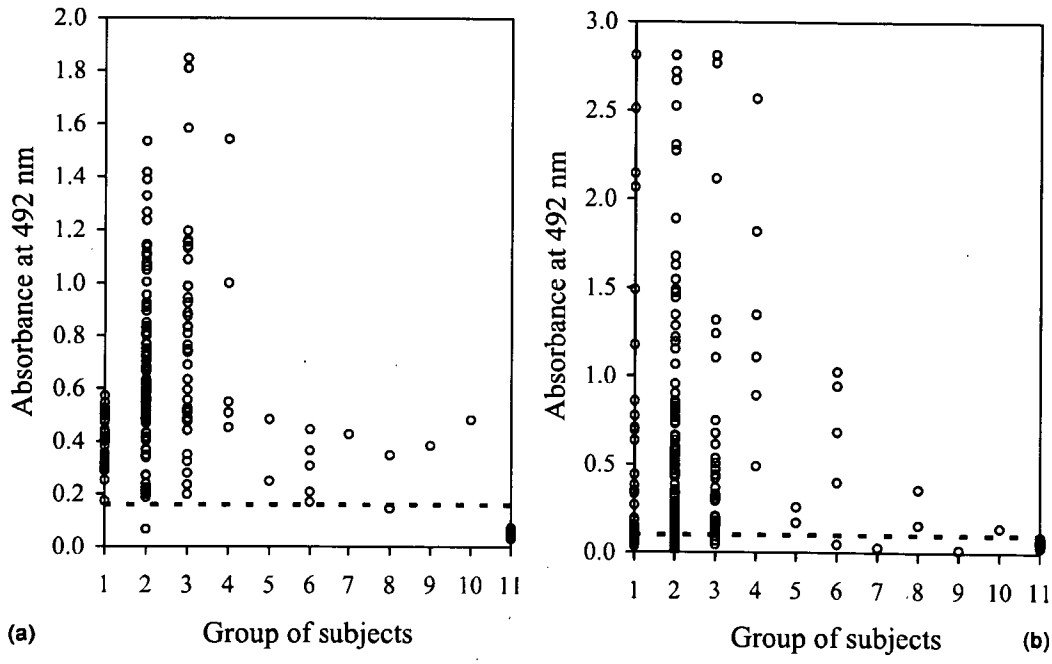

FIG. 1. The absorbances recorded in the ELISA used to measure serum levels of IgG (a) and IgG_4 (b) to *Opisthorchis viverrini*. Group 1 contained the 32 subjects who appeared parasite-free despite having no history of anthelmintic use. Groups 2 (98 subjects), 3 (32 subjects) and 4 (six subjects) contained the subjects who were only found positive for *O. viverrini*, excreting, respectively, 1–100, 101–1000 and >1000 eggs of this parasite/g faeces. Group 5 contained two subjects found positive only for *Strongyloides* larvae, Group 6 held five subjects found positive only for hookworm eggs, and Group 7 consisted of a single subject who was found positive only for the eggs of 'minute intestinal flukes'. Also included in the comparison were two subjects positive only for *Giardia* cysts (Group 8), one found positive only for *Taenia* eggs (Group 9), one positive only for *Sarcocystis* eggs (Group 10) and, as controls, 11 subjects from an area of Central Thailand where *O. viverrini* is unknown (Group 11). In each plot, the dotted line indicates the threshold for positivity in the ELISA.

generally low intensities of infection are probably attributable to praziquantel treatment of the relatively intense infections, which are more likely be symptomatic than the light infections.

There is considerable evidence indicating that opisthorchiasis could be diagnosed by the detection of specific antibodies. Antibodies to *O. viverrini* can be detected in the serum within 14 days of infection (Sripa and Kaewkes, 2000). Curiously, serum titres of anti-*O. viverrini* antibodies have been found to be higher in cases of cholangiocarcinoma than in patients with cholangitis caused by *O. viverrini* (or in uninfected individuals killed in accidents; Akai *et al.*, 1994; Itoh *et al.*, 1994).

If diagnosis could be based on urine samples, rather than on serum samples, it would be simpler and less painful and safer for the subject. Specific, anti-parasite IgG and/or IgG_4 can be found in urine samples of individuals infected with *Wuchereria bancropti*, *Leishmania*, *Cryptosporidium parvum*, schistosomes or *Fasciola*, although, compared with urine levels, the serum levels of such immunoglobulins tend to be more closely correlated with parasite burden (Gryseels *et al.*, 1995; Pospisilova *et al.*, 1997; Ramzy *et al.*, 1997; Snyman *et al.*, 1997; Itoh *et al.*, 2001). The mechanism of IgG release into the urine is unknown.

In the present study, with the results of faecal concentration used as the 'gold standard', the ELISA based on levels of