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Presence and gradual disappearance of filaria-specific urinary IgG4 in babies born to antibody-positive mothers: A 2-year follow-up study

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ABSTRACT

A total of 14 Sri Lankan pregnant women, who were anti-*Brugia pahangi* urinary IgG4 positive, and their 14 newborn babies were followed up for the urinary antibody for 2 years by enzyme-linked immunosorbent assay. Eight babies showed positive IgG4 reaction, at least once within 4 months after birth. Urinary antibody titers of mothers and their babies measured around the perinatal period showed a significant positive correlation, suggesting that baby's IgG4 was transferred from the mother through the placenta. The IgG4 decreased gradually and became negative in all positive babies by day 339.3 after birth. The present result provides a basis to judge if a positive urine ELISA test among babies is due to a new filarial infection.

1. Introduction

We reported an enzyme-linked immunosorbent assay (ELISA) that uses urine as samples to detect filaria-specific IgG4. The ELISA showed high sensitivity of 95.6% with *Wuchereria bancrofti*-infected people in Sri Lanka and specificity of 99.0% with non-endemic controls in Laos, Thailand and Japan [1]. Collection of urine samples is non-invasive and thus easily acceptable for most people. It is particularly useful in a study including young children. Using a specially devised plastic bag, mothers can collect urine from their babies, who do not show fear or uneasiness. In Sri Lanka, we studied filaria-specific urinary IgG4 in children aged less than 5 years old, and reported several positive babies born within 58 days [2]. Transfer of the antibody from mothers to their babies is the most likely explanation, and if so, it is important to determine how long the antibody will persist in urine, so that transferred IgG4 and newly generated one after filarial infection can be distinguished. In this paper, we report the result of a long-term follow-up study on the specific IgG4 levels in urine obtained from pregnant women and their newborn babies.

2. Materials and methods

Fourteen pregnant women were registered for the study. Twelve of them were urinary IgG4 positives before delivery and 2 were found positive after delivery. Except for one mother, urine samples were collected from them once or twice before delivery, and after delivery, both from the mothers and their 14 babies (8 males and 6 females) at 1, 2, 3, 4, 5, 6 and 12 months, and then at 2 years, as a rule. For various reasons, the dates of collecting samples could not be accurate. The treatment for filariasis was withheld during the study. For babies, a special plastic sampling bag with an adhesive collar (ATOM Pediatric Urine Collector; ATOM Medical, Tokyo) was utilized. The collar was to fix the bag on the skin around the urethral opening. Five milliliters of urine was transferred to a plastic bottle, mixed with sodium azide at 0.1%, and kept at 4 °C until the antibody was measured at Aichi Medical University School of Medicine, Japan.

Filaria-specific urinary IgG4 was measured by ELISA with a slight modification from our previous report [1,2]. In brief, crude antigens were prepared from adult *Brugia pahangi* females. A 96-well microtiter plate was coated with the antigens (5 μg/mL), and blocked with 1% casein buffer. Urine samples, without concentration, were applied to the plate (100 μL per well) and incubated overnight at 25 °C. After washing, 100 μL peroxidase-conjugated mouse monoclonal antibody to human IgG4, purchased from Southern Biotechnology Associates, Inc., AL, was reacted. The coloration was with ABTS peroxidase substrate (KPL Inc., Gaithersburg, MD). With the ELISA

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system, antibody levels were expressed as arbitrary units (U), ranging from 0 to 7290 U, and the cutoff value was 54.7 U [1]. To standardize the measurement of antibody titers, pooled and serially diluted sera from bancroftian filariasis cases were applied to each ELISA plate to construct a standard curve. Each urine sample was measured in duplicate and the average titer was used. A pair of mother and child urine was examined in the same plate.

The study proposal was reviewed by the ethical committees of University of Ruhuna, Sri Lanka and Aichi Medical University School of Medicine, Japan, and cleared. The mothers were explained on the purpose and methods of the study, and all participants gave their consent.

3. Results

The urinary IgG4 antibody of mothers fluctuated considerably during the 2-year period, and only 5 of 14 positive mothers showed consistently positive results (Fig. 1). Three borderline-positive mothers (mother–baby pair Nos. 5, 9, and 12 in Fig. 1) became positive only 1–3 times out of 8 measurements each after delivery. In an extreme case of fluctuation (pair No. 6), the mother's titers changed from 791 U to 14 U, and then to 1783 U, respectively, 35 days before, and 27 and 59 days after delivery. As a whole, however, ELISA results were highly consistent in terms of producing a positive reaction among mothers: by 6 months after delivery, a total of 84 samples were collected from 11 mothers (3 borderline positives excluded), and 80 of them (95.2%) gave positive reactions.

Eight of 14 babies (57%) showed a positive urine reaction, at least once within 4 months after birth. In 11 babies, IgG4 titers were constantly lower than those of mothers; in other words, 3 babies (pair Nos. 5, 6, and 8) showed, at one point, higher titers than their mothers'. All but 2 babies showed relatively high IgG4 titers 1–6 months after birth. In one exceptional baby (pair No. 2), IgG4 units were constantly 0 U in the first 6 months (186 days) despite relatively high titers in the mother, and in the other baby (pair No. 11), the titers were only 1 U at 1 month (30 days), and 0 Us thereafter. These 2 babies are

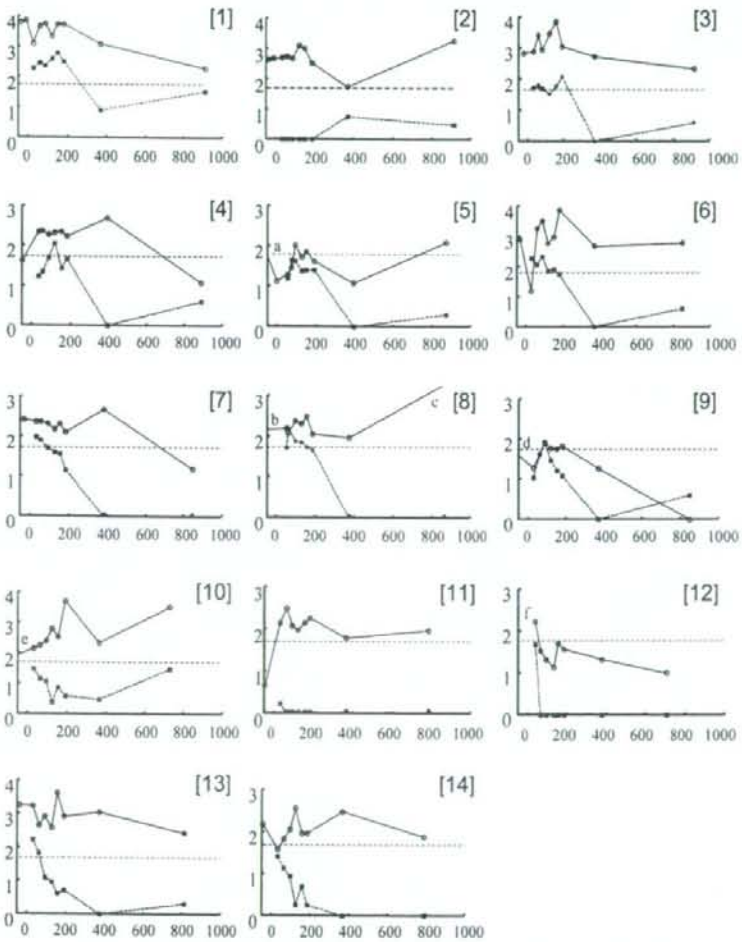

Fig. 1. Change in anti-*Brugia pahangi* urinary IgG4 titers in 14 mother–baby pairs. Pregnant women and their newborn babies were followed up for 2 years. Vertical axis: Log (IgG4 units +1). Note that the scales for Nos. 1–3, 6, 10, and 13 range from 0–4, and those for the other Nos. from 0–3. Horizontal axis: days before or after delivery (day = 0). [ ]: mother–baby pair number, dotted line: cutoff level, ○: mother, ■: baby. [a]IgG4 titer was 73 at day −61, [b]143 U at day −83, [c]1775 U at day 850, [d]56 U at day −95, [e]80 U at day −159, and [f]IgG4 was not measured before delivery.

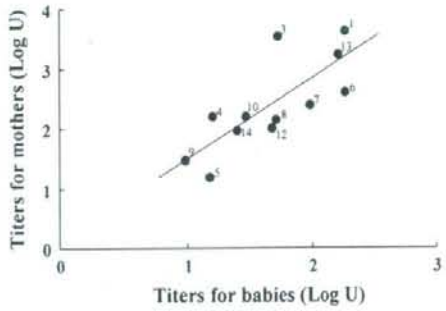

Fig. 2. Correlation of IgG4 levels in urine between mothers and their babies. An attached number to each dot corresponds to that for a mother–baby pair in Fig. 1.

considered different from the others in terms of 'completeness' of negative reaction.

In order to obtain an evidence for transplacental transfer of IgG4 antibody, perinatal IgG4 units of mothers and their babies were analyzed for correlation. In this case, the mother's unit is the average of 2 measurements nearest to the delivery day, and the baby's unit is the first measurement after birth. The analysis, excluding pair Nos. 2 and 11, revealed significant positive correlation ($r=0.770$, $P<0.004$), indicating that babies' IgG4 titers were dependent on those of mothers (Fig. 2).

After birth, babies' titers decreased gradually. All of 8 IgG4 positives became negative within a year (Fig. 1). The number of exact days required for negative conversion was estimated by simple interpolation using two data sets of IgG4 titer (U) and the number of days after birth, immediately before and after the final negative conversion. The antibody turned negative between 65.7 and 339.3 days, and the negative conversion rates of 50% and 80% were estimated at days 143.5 and 222.2 respectively (Kaplan–Meier method). The numbers of days required for negative conversion tended to be positively correlated with IgG4 levels of mothers, though this was not significant ($r=0.635$, $P<0.091$).

4. Discussion

Despite frequent samplings both from mothers and their babies for a period of 2 years, the mothers were consistently cooperative for the study. A most likely reason for the cooperation was the simplicity and non-invasiveness of urine collection. Blood collection from babies is often a troublesome and uncomfortable experience for mothers, especially in this type of study that does not have direct merit for them.

The present study confirmed a significant amount of filaria-specific IgG4 in urine among neonates born to the antibody-positive mothers. In addition, the titers of mothers and their babies correlated positively. Using serum samples, a positive correlation between antibody concentrations in mothers and their infants was considered as an irrefutable evidence for materno-fetal transfer of antibodies [3]. Our result with urine could be understood in the same way, though we did not encounter similar studies with urine only. IgG antibodies can be transferred from mothers to their fetuses by an active transport mechanism involving the neonatal Fc receptor for IgG (FcRn) expressed in the placenta [4]. The transfer of IgG subclasses was reported to be dependent on the terms of gestation [5,6], and levels of maternal IgG subclasses [6], and influenced by an infection like placental malaria [7], and birth weight of neonates [8]. As for IgG4, a cord concentration was reported to increase by gestational age, and reach the level of mother's serum by 36–37 weeks [6]. Thus, it will be possible that specific urinary IgG4 titers of babies become close to those of their mothers. In the present study, IgG4 was undetected after birth in 2 babies, even when the mothers had relatively high IgG4 titers, suggesting that appearance of IgG4 in urine involves complicated unknown mechanisms. The *in utero* sensitization of fetuses by filarial antigens would occur [9–11], but the effect on this study is unknown.

Transferred IgG disappears gradually after birth, and the speed of disappearance is reported different by species of infectious agents. For example, antibodies to mumps became undetected earlier than measles [12]. Our present study revealed that filaria-specific urinary IgG4 became negative by day 339.3. With babies born to *Onchocerca volvulus*-infected mothers, specific cord blood IgG4 at birth was reported strongly reduced 12 months after birth [11]. In applying urine-based ELISA to very young children, it is necessary to judge if a positive IgG4 reaction is due to transferred antibodies from an infected mother or a new filarial infection. Based on the present study, positive IgG4 at 1 year old and above can be regarded as filarial in Sri Lanka. All but one mother breast-fed the babies. Milk contains maternal antibodies including IgG4, but, in humans, they do not enter infant circulation [13].

In the present study, IgG4 levels of the same mothers were followed up for 2 years, and this will be an opportunity to consider the fluctuations, which could show tremendous ups and downs as observed in the pair No. 6. Possible factors causing fluctuations could be (i) time of the day to collect urine, (ii) chemicals in urine whose concentrations can be changing according to ingested foods, drinks, medicines, etc., (iii) urinary infections, and (iv) other factors, including pathology caused by filarial parasites. In relation to the factor (i), which would reflect, in part, hourly fluctuation of urine concentrations, we reported that timing of urine collection did not influence ELISA results, in terms of deciding positive or negative result [1]. With commercial urine ELISA kits for *Helicobacter pylori* infection, random single-void urine was reported suitable for IgG measurement [14]. As for the factor (ii), 28 different chemicals, including glucose, amino acids, caffeine, atropine, and acetaminophen, were tested with the same *H. pylori* ELISA, and reported to have no effect on titers [14]. However, using our IgG4 ELISA, we have observed opaque precipitation at the bottom of ELISA well in 24 of 10,409 (0.23%) urine samples collected in China [15], suggesting the presence of unknown substance in urine. Concerning (iii), the presence of microorganisms in urine and pH of urine did not influence urine *H. pylori* ELISA [16]. The pathology caused by filariae could influence urinary IgG4 titers. Chyluria and hematuria, which will indicate the leak of plasma into urine, are common symptoms of filariasis and tend to repeat remission and exacerbation. In this study with asymptomatic women, no trial was made to investigate the leak into urine. Technical variability is inevitable, but this could be minimized with utmost care: filaria-specific urinary IgG4 titers determined twice approximately 70 days apart in Thailand with 67 individuals showed a very good stability [17].

The present study confirmed the materno-fetal transfer of filaria-specific IgG4 and its disappearance from urine within a year. The information will enhance usefulness of the urine-based ELISA in the epidemiology and control of lymphatic filariasis, where young children are indispensable as a sentinel population to know filarial transmission.

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Distribution of filarial elephantiasis and hydrocele in Matara district, Sri Lanka, as reported by local leaders, and an immunological survey in areas with relatively high clinical rates

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ABSTRACT

To eliminate lymphatic filariasis by means of mass drug administration, it is essential to have reliable data on the disease distribution and prevalence in targeted areas. In Matara district, Sri Lanka, self-administered questionnaires were mailed to 2105 local leaders questioning the presence and the numbers of elephantiasis and hydrocele cases. The information provided by them revealed that elephantiasis was clearly aggregated in the southern part of the district along the coast, while hydrocele was distributed rather evenly in the whole district, including Deniyaya region where no endemic filariasis had been known. To confirm active transmission of filariasis in Deniyaya, *Wuchereria bancrofti* antigen and filaria-specific urinary IgG4 antibody were measured with 2436 subjects. The positive rates for antigen and antibody were 0.6% and 4.3%, respectively. The titer analysis of IgG4 according to age revealed that the youngest IgG4 positive was 3 years old, and that in 10 years old or less, there were 16 positives out of 607 children examined (2.6%). It was concluded that filarial transmission at a low level was going on in the region.

1. Introduction

For a national lymphatic filariasis elimination program based on mass drug administration, it is essential to have information on the distribution and prevalence of infection. In order to obtain the information as quickly and cost-effectively as possible, a variety of methods have been tested and applied in different epidemiological settings. These methods are referred to as rapid assessment procedures (RAPs). The RAPs include, for example, filarial antigen detection using commercially available immunochromatographic tests at systematically sampled points [1,2], or with a targeted subpopulation like schoolchildren [3], detection of obvious clinical signs such as elephantiasis and hydrocele [4], and vector mosquito surveys for filarial infection [5–8]. Although scientific accuracy might be sacrificed to various extents, the clinical signs have been utilized to obtain necessary information to plan and execute a filariasis elimination program, especially in areas with limited information on the disease. A hydrocele rate based on examination by a physician was reported useful to estimate a level of endemicity, as the rate correlated well with a community microfilaria rate [9]. A more simple survey based on self-administered questionnaires was also shown to be useful, in which local leaders were questioned on the presence and the numbers of elephantiasis and hydrocele [4].

In Sri Lanka, it has been reported that bancroftian filariasis is endemic in the 'filarial belt' that lies along southwestern and southern coasts of the island country. Although the belt was described by Abdulcader and Sasa in their map as a zone of about 10 km in width [10], the boundary of the belt is vague and only a limited number of surveys were done outside the belt. Therefore, it is necessary to clarify if the infection is occurring in inland areas, and how far it extends, if any. In the present study, we mailed a questionnaire to local leaders of Matara district, and collected information on the presence and the numbers of elephantiasis (including lymphedema) and hydrocele cases in their territories of responsibility. The reliability of this method has been confirmed in our separate studies in Sri Lanka, in which the estimates of community hydrocele prevalence based on village leaders' information showed a significant positive correlation with the clinical and immunological estimates by clinicians (Yahathugoda et al., unpublished). After obtaining the information, and the data having been mapped out, a field survey was carried out in inland villages, where no filarial infection had been recorded before but the leaders reported relatively high rates of clinical cases. Schoolchildren and villagers were examined by Og4C3 ELISA for *Wuchereria bancrofti* antigen, and by ELISA for filaria-specific

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urinary IgG4 antibodies, with the purpose to determine if transmission is actually occurring in those areas.

2. Materials and methods

2.1. Study areas and subjects

The study was carried out in Matara district, located southernmost part of the country, facing the Indian Ocean. The district occupies the land area of 1290 km^2 and had the registered population of 761,236 in 2001 (Government census, 2003). The inland areas are hilly or mountainous, in general, and tea plantations cover a large part of the land. The district has 16 administrative divisions each administered by a Divisional Secretary. The 16 divisions are further divided into 650 *Grama Niladari* (GN) divisions. In each GN division, there are 3 categories of local leaders. They are *Grama Niladari*, *Samurdhi Niyamaka*, and *Govi Niyamaka*. There are 1 *Grama Niladari*, 1–3 *Samurdhi Niyamakas*, and 1 *Govi Niyamaka* in a GN division, as a rule. The *Grama Niladari* is the Village Headman and his/her duties include monitoring of census, electoral registers, housing and population data, and amenities of the community. He/she also keeps records of epidemics and accidents, and attends to general administration. The *Samurdhi Niyamaka* administers the disbursement of monthly allowances to poor families given by the government that helps rural communities to develop their economy and improve personal hygiene. The duties include activities on water supply, power supply and roads. The *Govi Niyamaka* assists communities in all matters pertaining to agriculture. The local leaders in the 3 categories are appointed government employees placed under the supervision of District Secretary (Government Agent). All local leaders joined the questionnaire-based survey, which took place in September–November, 1999.

An immunological survey was carried out in Feb., 2002 in 14 GN divisions (total population: 29,181) in Deniyaya region (Fig. 1A), which is the northernmost part of Matara district (about 40–50 km from the coast) and a tea plantation area in mountains. Fifteen schools along main roads were visited in the morning and blood and urine samples were collected from as many children as possible. They were students of grades 1 to 12, whose ages are between 6 and 18 years, as a rule. A community located close to each school was also visited in the afternoon and the residents excluding schoolchildren were examined. Both urine and blood samples were collected from a total of 2436 people.

Before the field visit, the study plan was reviewed by the ethical committees of University of Ruhuna (Sri Lanka) and Aichi Medical University (Japan), and approved.

2.2. Questionnaire distribution and data management

The questionnaire form in local language, which had been pretested for easiness in use and clarity, included several identification tags and 9 simple questions. Main questions were on the presence and the numbers of elephantiasis and hydrocele cases. Some economic indicators such as types of housing and toilet were also questioned, but not analyzed in this study. The 3 categories of local leaders were requested by District Secretary to cooperate on the questionnaire survey. A questionnaire form in an envelope was then mailed to each local leader, together with a reply paid envelope. Three weeks after posting, the second questionnaire form was mailed to all non-respondents, and the same was repeated after another 3 weeks.

Of 1730 replies received, 62 were abandoned due to unclear ID tags. With remaining 1668 replies, 647 GN divisions could be identified. As expected, numerical information given by leaders from the same GN division showed considerable inconsistency. To deal with this problem, detailed criteria were made how to compute representative figures for each GN division. For example, for the questions relating to population, housing and economic indicators, answers given by a *Grama Niladari* were used, and without his/her reply, answers by a *Samurdhi Niyamaka* or a *Govi Niyamaka* were substituted in this order. For the question on the numbers of elephantiasis and

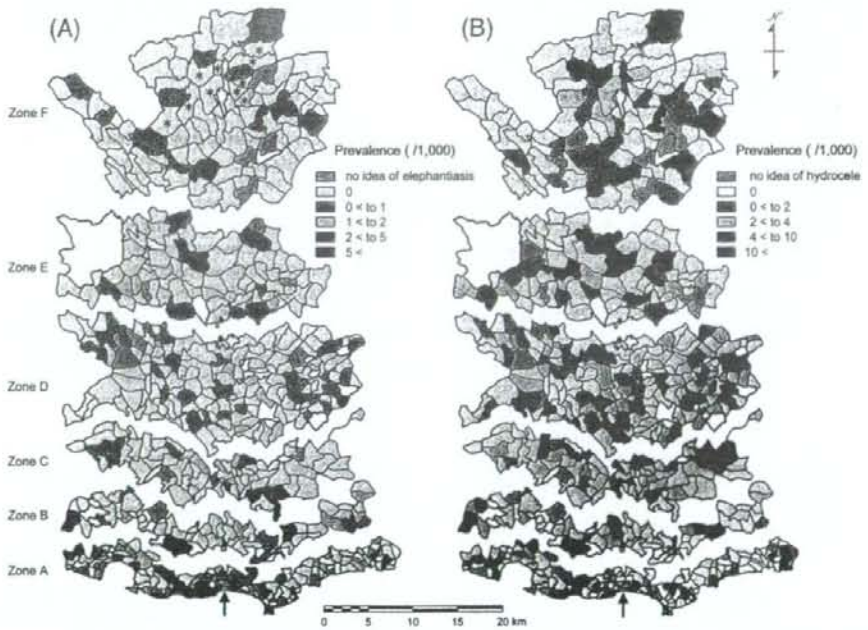

Fig. 1. Elephantiasis (A) and hydrocele (B) prevalence maps according to distance from the coastline. GN divisions with * in Fig. 1A are those selected from Deniyaya region for the immunological study. An arrow indicates the location of Matara City.

Table 1
The number of GN divisions reporting elephantiasis analyzed by prevalence level and distance from the coast

Prevalence level (/1000)	No. of GN divisions (% of all zones)						
	Zone A	Zone B	Zone C	Zone D	Zone E	Zone F	All zones
0	54 (13.9)	35 (9.0)	49 (12.6)	114 [29.3]	56 [14.4]	81 (20.8)	389 (100)
(a) 0 < to 1	27 (28.4)	11 (11.6)	8 (8.4)	28 (29.5)	8 (8.4)	13 (13.7)	95 (100)
(b) 1 < to 2	12 (37.5)	5 (15.6)	4 (12.5)	7 (21.9)	3 (9.4)	1 (3.1)	32 (100)
(c) 2 < to 5	11 (64.7)	2 (11.8)	3 (17.6)	1 (5.9)	0 (0.0)	0 (0.0)	17 (100)
(d) 5 <	10 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	10 (100)
(a)+(b)+(c)+(d)	60 [52.6]	18 [34.0]	15 [23.4]	36 [24.0]	11 [16.4]	14 [14.7]	154 [28.4]
All levels	114	53	64	150	67	95	543

Zones A, B, C, D, E, and F are, respectively, 0 ≤ to <3, 3 ≤ to <6, 6 ≤ to <10, 10 ≤ to <20, 20 ≤ to <30, and ≥30 km from the coast.
[ ] : % of GN divisions reporting elephantiasis cases, i.e., 100×{(a)+(b)+(c)+(d)}/All levels.

hydrocele, figures given by a *Grama Niladari* and a *Samurdhi Niyamaka* were averaged, and, without one of them, figures by a *Govi Niyamaka* were substituted. These and other detailed criteria were made considering different roles of leaders, and quality/reliability of information they could provide. The prevalence of clinical cases (per thousand) for each GN division was calculated as follows;

elephantiasis rate = 1000 × (reported number of cases)
÷(population of GN division provided by Grama Niladari)
hydrocele rate = 1000 × (reported number of cases)/(male population)

The male population was estimated as 50% of the total population.

2.3. Making a digitized map

A map of Matara district including all GN divisions is required to describe the distribution and prevalence of the clinical cases. A blueprint copy of hand drawn map, entitled "Matara District – Farmer Organizations and Agrarian Services Areas" and including 601 GN divisions, was provided by the District Secretariat of Matara. The scale is 1:63,360, and it was not known when the map was made.

The map was computerized with an image scanner, and then the data were imported into a geographic information system (GIS) using TNTmips (MicroImages, Inc., NE, USA). In the process of mapping, 86 GN divisions were abandoned, because their names did not match up to the GN divisions appearing on the hand drawn map. As the map was apparently old, new or renamed GN divisions could not be matched. Twelve areas in the hand drawn map were identified as forest or uninhabited land. Finally, the data from 561 GN divisions were included in the GIS map. To analyze the distribution and prevalence of elephantiasis and hydrocele according to the distance from the coastline, the map of Matara was sliced into 6 zones: zone A (0 to <3 km from the coast line), zone B (3 to <6 km), zone C (6 to <10 km), zone D (10 to <20 km), zone E (20 to <30 km), and zone F (≥30 km). In making a zoning based on a distance from coastline, many GN divisions were found covering 2 neighboring zones. In this case, the whole division was allocated to the zone further from the coastline. There were 12 GN divisions with a detached territory in a different zone. They were counted twice in analysis and thus the total number of data was inflated to 573.

2.4. Immunological survey

The antigen test was carried out using Og4C3 ELISA kit (TropBio Pty Ltd., Australia) with some modifications. Finger-prick blood was collected on a filter paper (Advantec Toyo, Japan), dried and kept at 4 °C until use. The filter paper was soaked in phosphate buffered saline and the soaking fluid was used as a sample. This filter paper ELISA showed almost identical efficacy compared with the ordinary Og4C3 ELISA using serum samples. The technical details are described elsewhere [11].

Urinary IgG4 was detected using ELISA reported previously [12] with slight modifications. In brief, a urine sample was collected in a plastic tube and mixed with sodium azide at 0.1% for preservation. Urine without concentration was added into a well of the microtiter plate pre-coated with female *Brugia pahangi* adult antigens (5 μg/ml), and incubated overnight at 25 °C. After washing, peroxidase-conjugated mouse monoclonal antibody to human IgG4 (Southern Biotechnology Associates, Inc., AL, U.S.A.) was added for 1 h at 37 °C. Coloration was with ABTS peroxidase substrate. Serially diluted positive serum samples were prepared for each microtiter plate to construct the standard curve, with which IgG4 levels were quantified using arbitrary antibody unit (U) ranging from 0 to 7290 U. The cutoff value was 54.7 U. The ELISA showed the sensitivity of 95.6% and the specificity of 99.0% [12].

3. Results

3.1. Distribution and prevalence of the clinical signs as reported by local leaders

A total of 2105 questionnaire forms were mailed to local leaders in the 1st round, and 1031 (49.0%) answers were obtained. In the 2nd round, 49.5% of previous non-respondents gave answers. The 3rd round resulted in much lower response (33.7%). In total, 1730 answers were obtained from 2105 addressees, with the final response rate of 82.2%. The population of 647 GN divisions identified in this study based on the questionnaire was 873,288. The distribution and prevalence of elephantiasis are shown in Fig. 1A, which clearly shows that

Table 2
The number of GN divisions reporting hydrocele analyzed by prevalence level and distance from the coast

Prevalence level (/1000)	No. of GN divisions (% of all zones)						
	Zone A	Zone B	Zone C	Zone D	Zone E	Zone F	All zones
0	57 (18.2)	28 (8.9)	41 (13.1)	81 (25.8)	45 (14.3)	62 (19.7)	314 (100)
(a) 0 < to 2	9 (17.6)	2 (3.9)	7 (13.7)	17 (33.3)	6 (11.8)	10 (19.6)	51 (100)
(b) 2 < to 4	9 [15.0]	10 [16.7]	6 [10.0]	22 [36.7]	7 [11.7]	6 [10.0]	60 (100)
(c) 4 < to 10	18 [25.7]	6 [8.6]	6 [8.6]	21 [30.0]	6 [8.6]	13 [18.6]	70 (100)
(d) 10 <	22 [46.8]	5 [10.6]	5 [10.6]	11 [23.4]	3 [6.4]	1 [2.1]	47 (100)
(a)+(b)+(c)+(d)	58 [50.4]	23 [45.1]	24 [36.9]	71 [46.7]	22 [32.8]	30 [32.6]	228 [42.1]
All levels	115	51	65	152	67	92	542

Zones A, B, C, D, E, and F are, respectively, 0 ≤ to <3, 3 ≤ to <6, 6 ≤ to <10, 10 ≤ to <20, 20 ≤ to <30, and ≥30 km from the coast.
[ ] : % of GN divisions reporting hydrocele cases, i.e., 100×{(a)+(b)+(c)+(d)}/All levels.

Table 3
Prevalence of *W. bancrofti* antigen and filaria-specific urinary IgG4 in selected GN divisions from Deniyaya region

GN division	No. exam.	No. Ag (+)	%Ag (+)	No. IgG4 (+)	%IgG4 (+)
Adaradeniya	125	0	0.0	4	3.2
Batandura North	184	2	1.1	5	2.7
Batandura South	85	0	0.0	0	0.0
Beliattakumbura	175	1	0.6	7	4.0
Deniyaya	215	3	1.4	22	10.2
Deniyaya West	153	0	0.0	5	3.3
Kalugalahena	238	3	1.3	16	6.7
Kotapola North	125	1	0.8	2	1.6
Morawaka	162	1	0.6	10	6.2
Pallegama North	233	2	0.9	11	4.7
Pathawita	183	0	0.0	4	2.2
Pussawela	180	0	0.0	7	3.9
Thanipita	202	0	0.0	6	3.0
Viharahena	176	2	1.1	5	2.8
Total	2436	15	0.6	104	4.3

elephantiasis is more confined to the coastal zone. Further, the number of GN divisions was analyzed according to zone and prevalence level in Table 1, in which 30 GN divisions had to be excluded because the village leaders answered that they had no idea of elephantiasis. The symptom was reported in more than a half (52.6%) of GN divisions in the zone A. The rate decreased gradually in inland zones and was 14.7% in the zone F. As for the GN divisions with 'high' prevalence, defined arbitrarily as >2 cases/1000 of population, 26 of 27 such divisions were in the zones A to C, within 10 km from the coastline. The distribution and prevalence of hydrocele are shown in Fig. 1B, and the GN divisions were analyzed by zone and prevalence level in Table 2. A total of 31 GN divisions were excluded because the leaders answered that they had no idea of hydrocele. The GN divisions with the highest prevalence (>10 cases/1000 of male population) were apparently more in the zone A than in the other zones, but the distribution of the symptom was more or less even in the whole district; the rates of GN divisions with hydrocele ranged from 50.4% in the zone A to 32.6% in the zone F, with the average of 42.1%.

3.2. Immunological study

A total of 2436 people in 14 GN divisions in Deniyaya region, where 8 elephantiasis and 49 hydrocele cases were reported, were examined for antigenemia and urinary IgG4 (Table 3). Of those, 15 (0.6%) were positive for antigen, and 104 (4.3%) were positive for antibody. By GN division, Deniyaya had the highest rates of antigen (1.4%) and antibody (10.2%), followed by Kalugalahena (1.3% and 6.7%). No correlation was found between infection rates (antigen and IgG4) and clinical rates (elephantiasis and hydrocele).

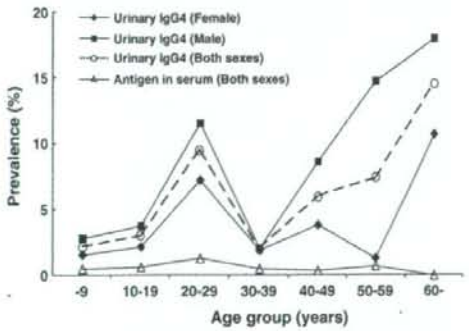

Fig. 2. Prevalence of *W. bancrofti* infection determined by Og4C3 ELISA and urinary IgG4 ELISA, and analyzed by sex and age group.

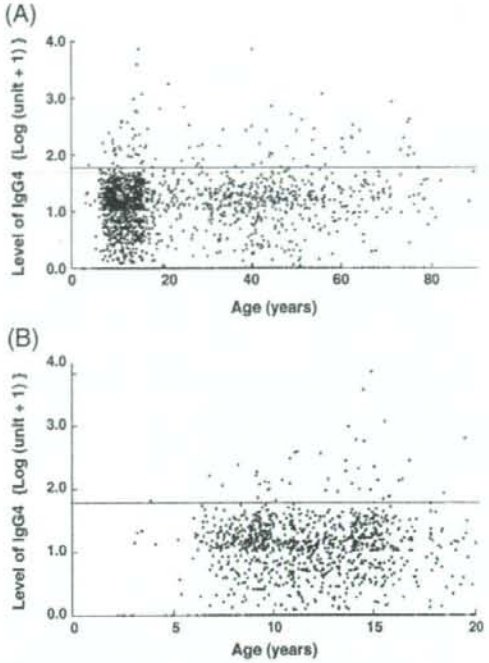

Fig. 3. Urinary IgG4 titers in the selected GN divisions from Deniyaya. (A) All people examined ($n=2436$), (B) People aged <20 years old ($n=1611$).

The prevalence of antigen and antibody was analyzed by age group and sex (Fig. 2). The prevalence of antigen was very low in all age groups, while that of urinary IgG4 showed an irregular pattern. In males, the age groups 20–29, 50–59 and 60-years showed higher prevalence than the age groups -9, 10–19 and 30–39 years ($P<0.03$ for all comparisons with χ^2 test). In females, the age group 20–29 years showed higher prevalence than the age group 10–19 years ($P<0.05$), and the age group 60- was higher than the age groups -9, 10–19 and 30–39 years ($P<0.05$). The oldest group (60-years) had the highest prevalence in both sexes (male: 18.0%, female 10.7%).

Urinary IgG4 levels are plotted against ages in Fig. 3. The youngest positive was a 3-year-old boy, with a low titer of 64.6 U. Under 11 years, all of 16 positive titers were 300 U or less, while in 11–19 years, 10 of 20 positive titers were more than 300 U ($P=0.007$, Fisher's exact test). At 14 and 15 years, 3 children had high titers over 1000 U (Fig. 3B).

4. Discussion

Two species of filarial parasite were recorded in Sri Lanka. Before 1940, filariasis was caused by *Brugia malayi*, with only exception of 2 towns, Galle and Matara, where *W. bancrofti* was endemic. Then, *B. malayi* started disappearing rapidly mainly because of deliberate removal of *Pistia*, the water plant required for breeding of *Mansonia* vectors, and had been eliminated mostly by mid 1960's. On the other hand, *W. bancrofti* gradually expanded its distribution along the coast, and after 1960's, it has become only significant species in the country [13,14]. Very old elephantiasis caused by *B. malayi* would not influence the present study, and hydrocele is a symptom of only *W. bancrofti*. *B. malayi* infection produces cross-reactive urinary IgG4, but it is unlikely that it interfered with the present IgG4 study in Deniyaya: the villages are mostly on mountain slopes, and there was no past report of endemic filariasis in the region.

Bancroftian filariasis in Sri Lanka was reported to be confined to a strip of land along the southwestern and southern coasts of the county [10]. However, a relatively recent report revealed some inland foci in Kurunegala, Gampaha and some other areas [14,15], suggesting a wider distribution of filariasis. To study the latest distribution of elephantiasis and hydrocele, we decided to mail questionnaires to local leaders, as Sri Lanka has reliable mailing services. In applying the questionnaire-based surveys, however, it is prerequisite to confirm the reliability of information given by local leaders. In order to verify this, we conducted a separate study in the neighboring district of Hambantota that has 576 GN divisions. A questionnaire was mailed to each *Grama Niladari* and collected data were analyzed as in the present study. Based on the results, GN divisions were categorized into the high-, medium- and low-endemic areas, and then, clinical surveys by doctors were carried out at 24 selected GN divisions (8 divisions/category), with a total of 1170 males of age ≥10 years. The 24 hydrocele rates obtained by doctors and the rates based on information by village leaders from corresponding GN divisions correlated positively ($r=0.55$, $P<0.05$) (Yahathugoda et al., unpublished). In yet another study in Galle district, questionnaire-based and urinary IgG4-based prevalence in 13 GN divisions resulted in a clear positive correlation ($r=0.78$, $P<0.01$) (Yahathugoda et al., unpublished). These results will indicate that local leaders can provide reliable information in terms of relative prevalence of hydrocele in Sri Lanka.

The surveys utilizing the postal services were successful in involving non-medical people as informers: 2105 questionnaires were mailed and 82.2% of addressees responded. The total population of Matara district reported by the leaders in 647 DN divisions was 873,288, which is higher than the official census result of 761,236 in 2001. This is probably caused by duplicate registration of families and individuals who were migrating, or changed residence for various reasons. For example, schooling outside the designated school area is common. This higher figure than expected would suggest, at least, that the *Grama Niladaris* did cover most of the people in the district. However, the reported figures of clinical cases seemed to be underestimates. In our previous clinical study in 3 suburban areas of Matara City (Polhena, Madihe and Walgama), the rates for elephantiasis/lymphedema and hydrocele were 3.0% and 6.2%, respectively [16]. In the present study, the GN divisions corresponding to the 3 study areas, though they are not exactly the same, showed the elephantiasis rate of 0.6% and hydrocele rate of 0.5%. The much lower prevalence is most probably due to reports of only obvious cases recognizable by people, and a tendency for a hydrocele patient to hide it. Similar gross under-reporting by key informants was reported in Ghana. However, both elephantiasis and hydrocele rates obtained through key informants and those in a clinical survey correlated well [17], indicating that people could provide reliable relative prevalence. In our present study, the information by local leaders revealed clearly an aggregated distribution of elephantiasis in the most endemic and long-standing filarial belt in Sri Lanka.

The questionnaire-based study revealed a clear contrast between the distributions of elephantiasis and hydrocele. It was a surprise to find so many reports of hydrocele cases in tea plantation areas on mountains, where no endemic filariasis had been reported. In order to confirm if filariasis transmission is actually occurring in these areas, an immunological survey was conducted in Deniyaya region, where relatively high clinical rates were reported. The antigen study with 2436 people revealed that the positive rate was very low (0.6%) and there was no difference in the rate by age group. From this result alone, active filarial transmission could not be confirmed in the region, because (i) the positives might get infection elsewhere, and (ii) false positive results were possible. The positive rate of urinary IgG4 was much higher (4.2%), and showed variation by age. The 20–29 year group had relatively high rates of 11.5% in males and 7.2% in females. Males of 50 years and above also had high rates. However, the prevalence in the 30–39 year group was very low and the reason was unknown. When urinary IgG4 titers were analyzed by age, a clear evidence of transmission was obtained, that is, (i) in 10 years old or less, there were as many as 16 IgG4 positives out of 607 examined (2.6%), and (ii) all of them had titers of ≤300 U, while 10 of 20 positives had >300 U in ages 11–19 years (Fisher's exact test, $P=0.007$), indicating the increase of titers in older children (Fig. 3B). Children are regarded as an important sentinel population to know recent transmission. Our studies in other endemic areas revealed the similar increase of IgG4 units by age among children [18,19]. We therefore concluded that filariasis transmission was going on in Deniyaya region at a low level.

Elephantiasis was more restricted in the filarial belt, while hydrocele was found widespread in the district. One possible explanation for different distribution could be as follows: In 3 suburban areas of Matara City, where microfilaria prevalence was 4.4%, we reported that the prevalence of lymphedema/elephantiasis increased lineally after 40 years of age, whereas, hydrocele increased lineally after 20 years [16]. As filariasis in Matara district was restricted inside the filarial belt until 1965 [10], the infection must have been spreading beyond the belt since then. Thus, the history of endemic filariasis in most inland areas is not longer than 40 years, which would not be enough for elephantiasis to make appearance.

It is a general recognition that RAPs based on finding out clinical cases are only effective when endemicity is high enough to produce many clinical cases [20]. In fact, the presence of a few clinical cases does not confirm the site of active transmission. In the present study, reports on a small number of clinical cases triggered an immunological survey in Deniyaya region, and revealed an active endemic focus. The combined use of questionnaire-based RAP and urine IgG4 ELISA would be a useful method to detect unnoticed foci, which could pose a problem in the final stage of the elimination program.

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Enzyme-linked Immunosorbent Assay to Detect Urinary Antibody Against Recombinant rKRP42 Antigen Made from *Leishmania donovani* for the Diagnosis of Visceral Leishmaniasis

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Abstract. We recently reported the production of the recombinant kinesin-related protein of *Leishmania donovani* with a molecular weight of 42 kd (rKRP42) and the value of the antigen in serum-based ELISA for the diagnosis of visceral leishmaniasis (VL). In this study, the rKRP42 antigen was validated with ELISA using urine samples (rKRP42 urine ELISA). The urine-based ELISA showed 94% sensitivity (108 positives among 115 VL samples) and 99.6% specificity (239 negatives among 240 non-VL samples). The sensitivity and specificity are almost similar to our previous results by ELISA with acetone-treated *L. donovani* promastigote antigen and direct agglutination test, both methods being done by use of urine samples. A comparison of the rKRP42 urine ELISA with the commercially available urinary antigen detection kit (KAtex) using 108 VL samples showed much higher sensitivity of the ELISA (96.3%) than KAtex (55.6%). The use of the rKRP42 antigen with urine samples will facilitate epidemiologic studies.

INTRODUCTION

Visceral leishmaniasis (VL) is caused by a protozoan parasite *Leishmania donovani* complex. In full-blown disease, the mortality rate ranges from 80% to 100% if not treated. Even with treatment, sometimes the case fatality rate reaches > 10%.[1] Recently, in a population-based study in Bangladesh, Ahlwalia and others[2] reported a VL incidence of 2% per year from 2000 to 2002, with a case fatality rate of 19% among adult women (≥ 15 years of age) and 6–8% among the other demographic groups (girls < 15 years of age and males of all ages). They also reported that the median duration of illness before receiving treatment was significantly longer in females than males. Delays in diagnosis and treatment increase the risk of complications and death.[1] VL is targeted for control under the neglected tropical diseases program.[3] The effective control of VL has been hampered because of the lack of suitable tools for early detection of infection and effective chemotherapy. Therefore, an accurate, easy, and low-cost diagnostic method is an urgent necessity.

Because of invasiveness and inadequate sensitivities, demonstration of the causative parasites in aspirates from lymph nodes, bone marrow, and the spleen has limited its use in a field survey.[4] In the last two decades, several serodiagnoses, like ELISA with crude or recombinant antigens,[5–7] and direct agglutinin test (DAT),[8,9] have been providing good diagnostic results. However, the use in endemic areas has been limited because of the expertise required for execution. In Bangladesh, DAT only has been available for clinicians at the Institute of Epidemiology, Disease Control and Research (IEDCR) situated in the capital, Dhaka. Recently, a recombinant antigen rK39, a part of *L. chagasi* kinesin-related protein, has been widely evaluated with ELISA or in dipstick format.[10,11] Although the antigen has been reported satisfactory in Indian subcontinent, the results varied considerably in different endemic areas. In India and Nepal,[12–14] the test showed the highest sensitivity of 100%, whereas it was significantly lower in Venezuela (88%),[15] in southern Europe (71.4%),[16] and in Sudan (67%).[17] The variation could be explained by differences in subspecies of *L. donovani* complexes, genetic differences in individual patients or in racial subgroups, and epidemiologic factors such as the length or severity of diseases.[18] Thus, it is desirable to develop new antigens for comparison. Recently, we reported the production of recombinant protein rKRP42, a part of *L. donovani* kinesin-related protein and a homolog to rK39, and evaluation of this antigen in ELISA with serum samples for the diagnosis of VL.[19]

Recently, the use of urine for blood has been considered valuable for the diagnosis of VL. A low-molecular-weight heat-stable leishmanial carbohydrate antigen was detected in urine by a latex agglutination test (KAtex).[20,21] We reported an ELISA with urine samples using acetone-treated *L. donovani* promastigotes antigen (acetone-treated urine ELISA),[22] and a direct agglutination test with urine samples (urine DAT)[23] for the diagnosis of VL, which showed, respectively, sensitivities of 95% and 90.7% and specificities of 95.3% and 97.3%. These figures are almost comparable to serum-based ELISA and DAT. In this study, we report the usefulness of rKRP42 antigen in a new urine ELISA and compare the results with other urine-based immunologic tests.

MATERIALS AND METHODS

Urine and serum samples. Most urine samples tested in this study were the same ones used in our previous study.[23] Additional samples from 44 VL patients and 17 Japanese healthy controls were included. A total of 115 urine samples from defined VL patients, collected from different medical college hospitals (96 from Rajshahi Medical College hospital, 11 from Mymensingh Medical College hospital, and 8 from Institute of Epidemiology, Disease Control and Research) in Bangladesh, were used to compute sensitivity of rKRP42 urine ELISA. VL was diagnosed in accordance with the WHO guideline of initiation of treatment.[24] Briefly, the diagnosis was based on well-defined clinical symptoms along with sup-

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portive hematologic features (anemia, leucopenia, reversed albumin globulin ratio, etc.) and was confirmed by parasitologic test or at least by one serologic test. The clinical symptoms included at least three of the following symptoms: intermittent chronic fever for > 1 month, splenomegaly, hepatomegaly, anemia, wasting, and lymphadenopathy. Malaria, tuberculosis, enteric fever, and other diseases that could produce similar clinical symptoms had been ruled out as much as possible. Among the 115 patients, 40 were confirmed parasitologically: Leishman-Donovan bodies were detected in smears of splenic aspirates (30 patients) or bone marrow aspirates (5 patients), and promastigotes were shown in 5 patients after inoculation of aspirate materials in Novy, MacNeal, and Nicolle medium. Of the other 75 clinically confirmed patients, 47, 9, and 19 were positive by conventional DAT, aldehyde test, and rK39 dipstick test, respectively. At the time of sample collection, patients were either in the course of treatment with sodium antimony gluconate at a WHO-recommended dose[25] or just before the initiation of treatment. A total of 240 non-VL urine samples were used for a specificity study. Fifty-nine control samples were taken from apparently healthy individuals with no past history of kala-azar from endemic areas in Bangladesh (endemic healthy control [EHC]). Eighty samples from Japanese individuals were used as non-endemic healthy controls (NEHC). Fifty-eight malaria samples collected in Solomon Islands, 13 tuberculosis samples from Bangladesh, 23 cutaneous leishmaniasis (CL) samples from Ecuador, and 7 samples from patients with other diseases were also included. Other diseases category included amebic liver abscess (2 cases), aplastic anemia (2), aplastic anemia with nephrotic syndrome (1), aortic stenosis (1), and viral fever (1). Immediately after collection of urine, NaN_3 was added to each sample at the final concentration of 0.1% as preservative, and the samples were transported to Japan at ambient temperature and kept at 4°C until immunologic tests.

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.

Effect of storage of urine samples on *L. donovani*–specific IgG titers. This study included urine samples that had been collected > 6 years before. To study the effect of storage of NaN3-added urine samples at 4°C, *L. donovani*–specific IgG titers of the same urine samples from 51 VL and 211 non-VL subjects were measured with acetone-treated urine ELISA at three different times: the first measurement with relatively fresh urine samples (collected within 1 year) was in February 2001, the second one in May 2003, and the third one in November 2007. The ELISA with acetone-treated *L. donovani* promastigotes antigen was described elsewhere.[22] The measurement (all in duplicate) was standardized using a set of serially diluted positive sera prepared for every microtiter plate. The sensitivities and specificities obtained in the three measurements were compared using the χ^2 test, and the geometric mean IgG titers were compared using paired t test.

The rKRP42 urine ELISA. The production of rKRP42 antigen was described elsewhere.[19] An ELISA was performed as follows. Flat-bottomed 96-well microtiter plates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 1 μg/mL (100 μL/well) rKRP42 antigen and incubated overnight at 4°C. After blocking with casein buffer (1% casein in 0.05 mol/L Tris-HCl buffer with 0.15 mol/L NaCl, pH 7.6) for 2 hours at room temperature, the wells were loaded with 100 μL of urine without concentration and incubated at 37°C for 1 hour. After four washes with PBS(–) (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 at 37°C for 1 hour. After washing four times, ABTS substrate (KPL, Gaithersburg, MD) was reacted for coloration for 1 hour at room temperature. The optical density was measured at 415 and 492 nm as reference. Each sample was assayed in duplicate, in which, if the absorbance values of the duplicates differ > 40% from their average, the sample was retested. Antibody levels were expressed arbitrarily as unit (U), which was estimated from the standard curve constructed for each plate with serially diluted positive sera. The cut-off point for anti-rKRP42 IgG was first calculated as the mean plus 3 SDs of log (unit + 1) values of the NEHC, and [antilog of (the mean + 3 SDs) – 1] was regarded as the cut-off unit.

Urine latex agglutination test (KAtex). The KAtex was performed with 108 VL (7 VL samples could not be tested because of inadequate quantity) and 240 non-VL urine samples according to the manufacturer's protocol (Kalon Biological, Aldershot, UK) except that 100 μL of urine was used for pretreatment of 5-minute boiling instead of recommended 250 μL to 1 mL. Before changing the volume of urine, we tested 10 urine samples and confirmed that the use of 100 μL did not influence the results. After bringing all the test reagents to ambient temperature, 50 μL of urine sample was placed to a reaction zone in the glass slide, and a drop of test latex was added to it. The liquids were stirred to a completely homogenous mixture and rotated continuously for 2 minutes. A negative control in the reaction zone next to the test sample was run each time. Any agglutination discerned compared with the negative control was considered positive.

RESULTS

Effect of storage of urine samples. Fifty-six VL and 211 non-VL urine samples stored at 4°C were tested with the acetone-treated urine ELISA in February 2001, May 2003, and November 2007, which showed sensitivities of 91.1%, 91.1%, and 82.1% and specificities of 95.3%, 97.2%, and 98.1%, in order of measurement (Table 1). There were no differences among the sensitivities (χ^2 test, 2 df, $P > 0.24$) and the specificities (χ^2 test, 2 df, $P > 0.23$). The mean IgG units in the first, second, and third measurements with the VL samples were 103.10, 83.86, and 61.11 U, respectively, and those with the non-VL samples were 0.93, 0.94, and 0.89 U, respectively (Table 1). In the VL samples, paired t test showed significant differences between the first and second means ($P < 0.012$), the second and third means ($P < 0.002$), and the first and third means ($P < 0.001$). Because the first measurement was done with relatively fresh samples, we considered its mean unit as 100% and calculated the percentages of IgG units in the second and third measurements. They were 81.3% and 59.3%, respectively (Table 1). The percentage reduction, when plotted on a graph, showed an approximate linearity, and a yearly rate of reduction was estimated to be 6.0% (40.7% reduction in 6.8 years). In the non-VL samples, no significant differences were obtained ($P > 0.28$ in all comparisons).

TABLE 1
Effect of storage of samples on sensitivity and specificity and on mean antibody unit with acetone treated urine ELISA

		Measurement time		
		2001 February	2003 May	2007 November
Mean antibody unit (%)	Sensitivity	91.1% (51/56)	91.1% (51/56)	82.1% (46/56)
	Specificity	95.3% (201/211)	97.2% (205/211)	98.1% (207/211)
	VL	103.10 (100.0)	83.86 (81.3)	61.11 (59.3)
	Non-VL	0.93 (100.0)	0.94 (101.2)	0.89 (95.5)

The rKRP42 Urine ELISA. A total of 115 VL and 240 non-VL samples were tested for IgG using rKRP42 antigen (Figure 1). With the NEHC group, the cut-off was determined as 57.9 U, based on which the test showed a sensitivity of 94.0% (108 positives among 115 VL samples) and a specificity of 99.6% (239 negatives among 240 non-VL samples). Of the 108 positive VL samples, only 10 had titers < 1,000 U. There was no difference between the sensitivities when calculated with the parasite-confirmed VL group (92.5% with 40 samples) and clinical VL group (94.7% with 75 samples; χ^2 test, $P > 0.64$). The specificities for EHC, NEHC, M, TB, and CL were 100%. Only one sample of other diseases category (aplastic anemia with nephrotic syndrome) became positive by the test.

KAtex. The urinary antigen detection assay KAtex showed a low sensitivity of 55.6% (60 positives among 108 VL samples) and a high specificity of 100% with 240 non-VL samples. Among the 108 VL samples, 36 were parasite positive, the rest (72) were clinical VL, and the KAtex positive rate for the former group was 63.9% (23 positives) and for the latter group was 51.4% (37 positives). No significance difference was obtained between the groups (χ^2 test, $P > 0.21$). In this study, 13 samples (of which 5 were parasitologically confirmed) were collected before initiation of treatment, and the rest (95 samples) were collected at various times in the course of treatment. Further analysis showed that the pre-treatment samples had a high sensitivity of 76.9%, whereas the treated samples had much lower sensitivity (44–54%; Table 2). However, the difference between the pre- and undertreatment (all combined) samples was not significant (χ^2 test, $P > 0.09$).

DISCUSSION

Because most of the urine samples used in this study were stored at 4°C for years, it would be essential to study the effect of storage on sensitivity, specificity, and the level of antibody unit, and for the study, acetone-treated urine

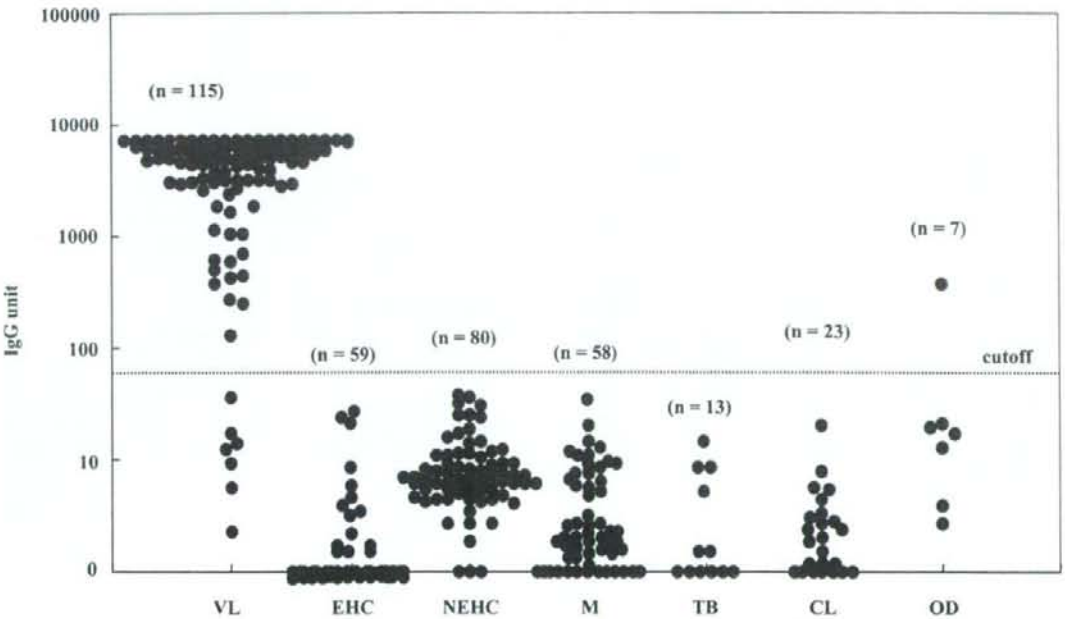

FIGURE 1. Detection of anti-rKRP42 IgG in urine of visceral leishmaniasis patients, healthy individuals, and controls with various diseases by ELISA. Each symbol (•) stands for a single urine sample. The horizontal dotted line represents the cut-off value: 57.9 U. VL = visceral leishmaniasis; EHC = endemic healthy controls; NEHC = non-endemic healthy controls; M = malaria; TB = tuberculosis; CL = cutaneous leishmaniasis; OD = other diseases; n = number of samples.

TABLE 2

KAtex-positive rate in relation to sample collection day with treatment

Sample collected on the day after initiation of treatment	Tested samples	KAtex positives
0 (before initiation of treatment)	13	76.9% (10/13)
1–10	9	44.4% (4/9)
11–20	15	53.3% (8/15)
21–30 or more	6	50.0% (3/6)
No clear history*	65	53.8% (35/65)

* In the patient's data sheets, the exact date of collection was not mentioned, but all the samples were collected within the course of treatment

ELISA was applied. The storage clearly decreased mean antibody units at a rate of 6% annually in the VL group. To the best of our knowledge, this is the first quantitative report on the reduction of *L. donovani*–specific antibody after long-term storage of NaN_3-added urine samples at 4°C. Thus, theoretically, urine storage could decrease sensitivity and at the same time might improve specificity. However, such effects will be only on borderline positive titers. In this study, up to 6 years and 8 months of storage did not affect the sensitivity and specificity at a statistically significant level.

The rKRP42 urine ELISA with samples kept for years showed a high sensitivity of 94%. The ELISA also resulted in a very high specificity of 99.6%. These results are comparable to our previously reported figures obtained by the acetone-treated urine ELISA, urine DAT, and rKRP42 serum ELISA. The commercially available rK39 dipstick test (In-Bios International, Seattle, WA) also gave similar results in our study in Bangladesh (Table 3).

When three different methods for urinary antibody detection (rKRP42 urine ELISA, acetone-treated urine ELISA, and urine DAT) were taken into consideration, a total 90.1% (64 of 71) VL samples were positive and 95.9% (211 of 220) of non-VL samples were negative by all three tests. Variable results, i.e., positive by one test but negative by other test(s), were obtained in 3 of 71 VL samples (4.2%), and 8 of 220 non-VL samples (3.6%). All these methods failed to detect four VL cases, of which three were parasite positives, or cross-reacted with one non-VL sample that was collected from a patient of aplastic anemia with nephrotic syndrome.

When we compared the rKRP42 urine ELISA with the rKRP42 serum ELISA and the rK39 dipstick test using 71 VL cases, 65 (91.5%) samples became positive and 4 (4.6%) samples were negative in all assays. Two samples from one parasite-confirmed and one clinical VL gave false-negative results with rKRP42 urine ELISA but were positives by rKRP42 serum ELISA. Of the four false negatives, three were collected from parasitologically confirmed patients. These three samples gave false-negative results with conventional serum-based DAT[23] and with urinary antibody detection assays (acetone-treated urine ELISA and urine DAT).

There is always a fraction of infected persons without clinical manifestations. Khalil and others[26] reported that, in eastern Sudan, the ratios of clinical and subclinical cases in Um-Salala village, respectively, in 1994/95 and 1995/96, were 1.2:1 and 2.6:1, and in Mashrau Koka village were 1:11 and 1:2.5. In another study conducted in Bihar state, India, 69% of asymptomatic seropositves by rK39 ELISA and dipstick developed kala-azar within 1 year,[27] suggesting that many of the asymptomatic cases were in a pre-clinical state. In predicting possible clinical cases, an ELISA, which is quantitative, would be

TABLE 3

Sensitivity and specificity of rKRP42 urine ELISA for the diagnosis of visceral leishmaniasis

Samples	Methods	Sensitivity*	Specificity†						References
		VL	EHC	NEHC	M	TB	CL	OD	Total	
Urine	rKRP42 urine ELISA	94.0% (108/115)	100% (59/59)	100% (80/80)	100% (58/58)	100% (13/13)	100% (23/23)	85.7% (1/7)	99.6% (239/240)	
	Acetone- treated urine ELISA	95.0% (57/60)	100% (59/59)	100% (53/53)	91.5% (54/59)	100% (13/13)	87.0% (20/23)	71.4% (5/7)	95.3% (204/214)	Islam and others (23)
	Urine DAT	90.1% (68/75)	96.6% (57/59)	100% (65/65)	100% (58/58)	92.3% (12/13)	87.0% (20/23)	71.4% (5/7)	96.4% (217/225)	Islam and others (24)
	KAtex	55.6% (60/108)	100% (59/59)	100% (80/80)	100% (58/58)	100% (13/13)	100% (23/23)	100% (7/7)	100% (240/240)	
Serum	rKRP42 serum ELISA	94.6% (70/74)		99.3% (148/149)					99.3% (148/149)	Takagi and others (19)
	rK39 dipstick test	93.2% (69/74)								Takagi and others (19)

* Number positive/total number of VL samples.
† Number negative/total number of non-VL samples in each category.
VL = visceral leishmaniasis; EHC = healthy controls from endemic areas; NEHC = healthy controls from non-endemic areas; M = malaria; TB = tuberculosis; CL = cutaneous leishmaniasis; OD = other diseases.

more advantageous than a dipstick format, that is, high antibody titers, or 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.[28] However, frequent collection of blood often hinders the field surveys. Urine samples that can be easily and safely collected will solve many of the difficulties experienced with blood collection in a field study, especially with children.

Antigen detection is more useful than antibody detection for diagnosing active VL cases. KAtex has been widely evaluated in many places such as in Nepal[29] and India[30] and found to have a sensitivity of 47.7% and 73.5%, respectively. In this study, the sensitivity and specificity of KAtex was 55.6% and 100%, respectively. Because the study included urine samples from clinically suspected cases and undertreatment cases, the sensitivity could be an underestimate. In our separate study, it was observed that six of seven KAtex-positive cases turned negative after completion of 28-day course of sodium stibogluconate treatment, suggesting its diagnostic and prognostic value.

The rKRP42 urine ELISA has been shown to be highly sensitive and specific. The stability of urinary antibody at 4°C even after prolonged storage has also been confirmed. Our rKRP42 urine ELISA would be a better alternative for the diagnosis of VL in a field survey, especially when repeated sampling is needed. KAtex that uses urine would be good adjunct for determining the active VL cases among rKRP42 urine ELISA positives.

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MOLECULAR DISCRIMINATION BETWEEN *PARAGONIMUS HETEROTREMUS* AND TWO FORMS OF *P. WESTERMANI* OCCURRING IN THAILAND

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Abstract. In areas of central Thailand where paragonimiasis is endemic, metacercariae of *Paragonimus westermani* (large metacercarial form) and *P. heterotremus* have been detected in a single crab species. Of these two species, only the latter has been confirmed to infect humans. In southern Thailand, we have previously identified another form of *P. westermani* (small metacercarial form) in another crab species, which also acts as host for *P. westermani* (large metacercarial form). In this study, we established a new multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage. We found that multiplex PCR in combination with restriction enzyme digestion (PCR-RFLP with *Bsa*HI) was effective for the discrimination.

INTRODUCTION

During an intensive field survey for lung flukes in southern Thailand, we found two forms of *Paragonimus westermani* metacercariae in a single crab species, *Phricotelphusa aedes* (Binchai *et al*, 2007; Sugiyama *et al*, 2007). Metacercariae of these two forms had the same shape, but were of different sizes: the diameter of metacercarial cysts of the large form is about twice that of the small one. As the nuclear ribosomal DNA (rDNA) second internal transcribed spacer (ITS2) sequences obtained from the large metacercarial form were identical to those of *P. westermani*, whose sequence was deposited in the GenBank/EMBL/DDBJ nucleotide databases under the accession number of AF159604 (referred to as *P. westermani* strain Thailand), we referred to the small metacercarial form as *P. westermani*-like for descriptive purposes.

In Thailand, human infections with *P. westermani* have not been confirmed, although *P. heterotremus* is known to affect humans (Srisont *et al*, 1997; Blair *et al*, 1998). The metacercariae of these two species have been detected in the same crab host in paragonimiasis-endemic areas (Miyazaki, 1991). Therefore, we had developed methods that could be used as reliable tools for discriminating these two lung fluke species. We demonstrated that multiplex PCR method was the most efficient because species identification involved a single round of PCR in a single tube (Sugiyama *et al*, 2005). In this study, we modified the previously established multiplex PCR method and evaluated its applicability for discriminating between *P. heterotremus* and two forms of *P. westermani* at the metacercarial stage.

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MATERIALS AND METHODS

Parasite material and DNA isolation

Metacercariae of *P. heterotremus* were harvested from the freshwater crab, *Larnaudia larnaudii*, captured in a mountain stream in Saraburi Province, Thailand (Kawashima *et al*, 1989). Metacercariae of *P. westermani* and *P. westermani*-like were isolated from the freshwater crab, *Phricotelphusa aedes*,

captured in a mountain stream in Surat Thani Province, Thailand (Sugiyama *et al*, 2007). This study also includes *P. siamensis*, the species known to be closely related to *P. westermani* (Blair *et al*, 1998). Metacercariae of *P. siamensis* were harvested from the freshwater crab, *Sayamia germaini*, captured in paddy fields in Prachin Buri Province, Thailand (Srisont *et al*, 1997). DNA samples were prepared from the metacercariae as previously described (Sugiyama *et al*, 2002).

DNA amplification and sequencing

For multiplex PCR amplification, we constructed new species-specific forward primers based on the respective rDNA ITS2 sequences in order to generate the products that would remain uncut by further restriction enzyme digestion. The sequences (and alignment positions) (Fig 1) of the primers used for the multiplex PCR are as follows:

1) Interspecies-conserved forward primer (3S): 5′ GGTACCGGTGGATCACT CGGCTCGTG 3′;

2) Interspecies-conserved reverse primer (A28): 5′GGGATCCTGGTTAGTTTCTTTTC CTCCGC 3′ (Bowles *et al*, 1995);

3) *P. heterotremus*-specific forward primer (PhTF2): 5′ CAAATCCGGGCGTAT CCATGTTGTG 3′ (positions 238 to 262);

4) *P. westermani*-specific forward primer (PwTF4): 5′ TCTGCGTTCGAT GCTGACCTACG 3′ (positions 368 to 390, a sequence common between the two forms of *P. westermani*).

These four primers were included in a single-tube reaction. Multiplex PCR amplification was performed using 0.1 μM of PhTF2 and PwTF4 primers, 0.5 μM of 3S and A28 primers, 2.5 units of the *Taq* polymerase (Invitrogen, USA) and 10 ng of DNA template. The resulting PCR products were separated by electrophoresis in 3% (w/v) agarose gels.

The amplicons were extracted from agarose gels and sequenced using the corresponding primers and BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in an automated sequencer (ABI310, Applied Biosystems). The sequence alignment and comparison were conducted using GENETYX-WIN (ver. 7.0, Software Development, Japan) program.

Restriction enzyme digestion of the multiplex PCR products (PCR-linked restriction fragment length polymorphism (PCR-RFLP))

Amplicons (4 to 10 μl) were also digested with five units of *Bsa*HI (New England Biolabs, USA) at 37 °C for 1 hour. The samples were then separated by electrophoresis in 3% (w/v) agarose gels.

RESULTS

Using multiplex PCR method with the new species-specific primers, two products were amplified from each of the metacercarial DNA samples of *P. heterotremus* (ca. 520 bp and 250 bp), *P. westermani* (ca. 520 bp and 125 bp), and *P. westermani*-like (ca. 520 bp and 125 bp) (Fig 2, lanes 1 to 3). However, a single 520-bp product was generated from the DNA samples of *P. siamensis* (Fig 2, lane 4). Sequence analysis of the amplification products (520 bp and others) revealed that the products corresponded to the rDNA ITS2 region of the respective species (Fig 1).

For species discrimination by RFLP using the multiplex PCR products, we selected restriction enzyme *Bsa*HI based on the putative restriction maps generated from ITS2 region sequences (Fig 1). Digestion of multiplex PCR products of *P. westermani*-like produced three fragments (ca. 270, 170 and 90 bp) from the 520-bp amplicon (Fig 2, lane 7). However, the 520-bp amplicons of the other 3 species (*P. heterotremus*, *P. westermani* and *P. siamensis*) produced two fragments (ca. 350 and 170 bp; Fig 2, lanes 5, 6 and 8). Multiplex PCR products of less than 520 bp in size (250-bp product for *P. heterotremus* and 125-bp products for *P.*

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Ph 001: TGTCGATGAAGAGCGCAGCCAACTGTGTGAATTAATGTGAACTGCATACTGCTTTGAACA 060
Pw 001: .....................................C..................... 060
PL 001: .....................................C..................... 060

Ph 061: TCGACATCTTGAACGCATATTGCGGCCACGGGTTAGCCTGTGGCCACGCCTGTCCGAGGG 120
Pw 061: ............................................................ 120
PL 061: ............................................................ 120

                          BsaHI
Ph 121: TCGGCTTATAAACTATCGCGACGCCCAAAAAGTCGCGGCTTGGGTTTTGCCAGCTGGCGT 180
Pw 121: ............C.....[......]..................................... 180
PL 121: .........G.........[......]..................................... 180

                                                        BsaHI
Ph 181: GATTTCCCCAACGTGGCCTTGTGTCTGTGGGGTGCCAGATCTGTGGCGTTTCCCTAACAA 240
Pw 181: ...C.......TC...T......C..................A................T 240
PL 181: ...C.......TC...T......C.....................[.....C].....T 240

          PhTF2-->
Ph 241: ATCCGGGCGTATCCATGTTGTGGCTGAAAGCCTTGATGGGGATGTGGCAACGGAGTCGTG 300
Pw 241: .CT..C...C.C...C....C..............C.................A..... 300
PL 241: .CT......C.C...C....C..............C.................A..... 300

Ph 301: GCTCAGTGAATGATTTATGTGCACGTTCCGCTGTCCCGTCATCATCTATGGTTGAAGTTG 360
Pw 301: .......A..............G....T........T...T......G.....C.T.... 360
PL 301: .........T..G.........G.............T...T......G.....T.T.... 360

Ph 361: CGCGTGGTGTG--TCCGATGCTGACCTATATATGTGCCATGTGGCTCATTTTCCTGACCT 420
Pw 361: ........C..CG.T.............CG..............TC....C.T....... 420
PL 361: ........C..CG.T.............CG..............T.....C......... 420
                   PwTF4-->

Ph 421: CGGATCAGACGTGAGTACCCGCTGAACTTAAGCATATCACTAA 463
Pw 421: .....T..................................... 463
PL 421: ........................................... 463
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Fig 1- Sequences alignment of the ITS2 region from *P. heterotremus* (Ph), *P. westermani* (Pw) and *P. westermani*-like (PL) metacercariae. The 5' and 3' ends of the sequences include 5.8S rDNA and 28S rDNA, respectively. A dot in the *P. westermani* and *P. westermani*-like sequences indicates identity with *P. heterotremus* sequence. The locations of the *P. heterotremus*-specific forward primer (PhTF2; 5' CAAATCCGGGCGTATCCATGTTGTG 3') and *P. westermani*-specific forward primer (PwTF4; 5' TCTGCGTTCGATGCTGACCTACG 3') are underlined. The recognition sites of *Bsa*HI (GR/CGYC) are located in boxes. The numbers refer to the alignment positions.

westermani and *P. westermani*-like) remained undigested by *Bsa*HI.

DISCUSSION

We previously reported that the multiplex PCR method we developed (Sugiyama *et al*, 2005) was effective for discriminating among the five *Paragonimus* species occurring in Thailand when used in combination with *Scr*FI digestion (Sugiyama *et al*, 2006). However, this method was not applicable for discriminating among *P. heterotremus* and two forms of *P. westermani* because the latter two forms showed identical PCR-RFLP patterns. Therefore, in this study, we treated the amplicons with *Bsa*HI chosen based on the sequence differences between these two forms of *P. westermani*. In addition, new species-specific primers were constructed to generate products that would remain uncut by *Bsa*HI digestion. The improved method was shown to be effective in discriminating among *P. heterotremus* and two forms of *P. westermani*.

Two forms of *P. westermani* were found

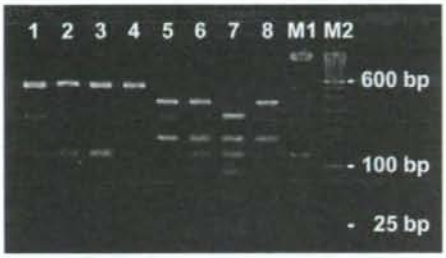

Fig 2- Multiplex PCR and multiplex PCR plus PRLP analysis of ITS2 amplification products from the metacercarial DNA samples of *P. heterotremus* (lanes 1 and 5), *P. westermani* (lanes 2 and 6), *P. westermani*-like (lanes 3 and 7) and *P. siamensis* (lanes 4 and 8). After digestion of the multiplex PCR products with *Bsa*HI, three bands were observed for *P. heterotremus* (ca. 350, 250 and 170 bp, lane 5), three bands for *P. westermani* (ca. 350, 170 and 125 bp, lane 6), four bands for *P. westermani*-like (ca. 250, 170, 125 and 90 bp, lane 7) and two bands for *P. siamensis* (ca. 350 and 250 bp, lane 8). Both the 25-bp and 100-bp DNA ladders were used to estimate the sizes of the bands (lanes M1 and M2, respectively).

to occur in Surat Thani, southern Thailand, and they both used a single crab species as the second intermediate host (Sugiyama *et al*, 2007). Possible discovery of *P. westermani*-like metacercariae were reported from crabs occurring not only in Surat Thani (Shibahara *et al*, 1995) but Nakhon Si Thammarat (Tsuzuki *et al*, 1995), the neighboring province of Surat Thani. However, little attention has been paid to *P. westermani*-like and its infection of humans has not been determined. To obtain accurate epidemiological information about the prevalence of the lung fluke species and forms in Thailand, various methods for identification are needed on parasitological materials obtained from host animals. The method developed in this study has the potential for this purpose.

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