Helminth infections, allergic disorders and immune responses: studies in Indonesia
Wahyuni, Sitti

Chapter 2

Comparison of an
enzyme linked immunosorbent assay (ELISA)
and a radioallergosorbent test (RAST)
for detection of IgE antibodies to *Brugia malayi*

Sitti Wahyuni [1,4]
Ronald van Ree [2]
Andarias Mangali [1]
Taniawati Supali [3]
Maria Yazdanbakhsh [4]
Erliyani Sartono [4]

[1]Department of Parasitology, Medical Faculty, Hasanuddin University, Makassar, Indonesia, [2]Department of Immunopathology, Sanquin Research,Amsterdam, The Netherlands, [3]Department of Parasitology, Medical Faculty, University of Indonesia, Jakarta, Indonesia and [4]Department of Parasitology, Leiden University Medical Centre, Leiden, The Netherlands

Abstract

The enzyme linked immunosorbent assay (ELISA) for specific IgE antibodies to *Brugia malayi* was compared with the radioallergosorbent test (RAST) for use in immunoepidemiological studies of lymphatic filariasis. Sera used were from individuals (aged 5-82 years) living in an area endemic for lymphatic filariasis in South Sulawesi, Indonesia. The percentage of positive IgE ELISA reactions (52.6%) among the population was lower than the percentage of positive RAST (94.5%). Although an overall significant concordance was found between the two assays (P<0.001), 328 (42.7%) individuals with a positive RAST result were negative in the ELISA, whereas only 6 (0.8%) subjects were positive by ELISA, yet negative by RAST. When the population was divided into those with active infection (positive for anti-filarial IgG4) and those not infected (microfilariae-negative and negative for anti-filarial IgG4), the correlation between the two tests was higher in the IgG4-positive (*rho*=0.70) than in the IgG4-negative (*rho*=0.52) group. These results indicate that in assessment of *B. malayi* specific IgE antibody RAST is superior to ELISA. However, given the use of radioactivity in the RAST method and our results obtained in subjects with high anti-filarial IgG4, one could consider using the IgE-ELISA in areas with high endemicity for filariasis. In areas with low endemicity or where control programs are implemented, sera will have to be tested by RAST.

Introduction

Lymphatic filariasis is a disease caused by nematode parasites of *Wuchereria* and *Brugia* species which affect more than 120 million people in tropical and subtropical areas of the world. As in many helminth infections, the humoral immune response against lymphatic filariasis is associated with high levels of immunoglobulin (Ig)E, not only restricted to parasite specific IgE but also covering non-specific polyclonal activation of IgE synthesis [292;293]. In humans, IgE elicits cell mediated reactions upon exposure to antigen. These reactions can be triggered by cross linking of either high-affinity IgE receptors present primarily on mast cells leading to immediate hypersensitivity or low affinity IgE receptors on eoshinophils and basophils resulting in release of bioactive cell contents and inflammation [294-296].

IgE is thought to play an important role in protective immunity and pathogenesis of helminth infections [72]. The initial indication that IgE may correlate with disease pathology for human lymphatic filariasis came in 1981 with the advent of the solid phase radioimmunoassay (SPRIA) now known also as RAST for quantization parasite specific IgE and the demonstration that patients with tropical pulmonary eosinophilia and elephantiasis had high level of IgE antibodies [73;297]. It is now also thought that the ratio of IgG4 to IgE in particular is important in disease progression following infection.

A so called "modified Th2" response characterized by high IgG4/IgE seems to be associated with asymptomatic helminth infections [66]. Therefore, interest in studying IgE antibodies in the context of immunity or pathology in populations living in areas where helminths are endemic is still strong and it is important that accurate tests are utilized. In the field of allergy, RAST is the standardized method to detect IgE responses to allergens. However, in epidemiological studies of helminth infections often the ELISA has been used to detect anti-parasite IgE. In schistosomiasis and in intestinal helminth infections, studies that have shown that protection is associated with high levels of IgE [92;298;299], have used the ELISA method. ELISA is indeed a cheaper, easier to use and less time-consuming method, criteria most important for use in tropical settings. Furthermore, the use of radioactivity in the RAST is a serious obstacle in its more widespread use.

A wide range of reagents for the measurement of IgE in ELISA have become more generally available and their performance needs to be compared again with RAST. So far only one study carried out two decades ago has compared a RAST with the ELISA technique for quantification of human IgE against soluble adult antigen of *B.malayi* [300]. It was demonstrated that the reported ELISA was ten times less sensitive in the measurement of IgE than a RAST. However, this study was restricted to seven samples and its main aim was to detect IgE in sera, which were negative when tested by ELISA. Other studies in helminth infectious diseases like hydatidosis [301] and schistosomiasis [302] have also compared both methods, but the number of samples have always been very limited.

The present study was designed to compare *B. malayi*-specific IgE titers of 768 individuals living in an endemic area for brugian filariasis when measured by ELISA or by RAST. Within this large population the performance of the two tests was compared in individuals with active infection and those free of infection, using anti-filarial IgG4 as a marker of active infection.

Material and methods
Study population
The study population consisted of life-long residents of the villages Salubarana and Kalia, Karossa district, South Sulawesi, Indonesia, an area where *B.malayi* is endemic. The study was approved by the Commission of Medical Ethics of Hasanuddin University. In cooperation with the head of the village and the health workers of the local District Health Center, all inhabitants were informed about the study and invited to participate. Informed consent was obtained from all participants prior to enrollment in the clinical, parasitological and venous blood collection study in accordance with the guidelines of Indonesian Department of Health and Human Services.

Detection of microfilariae

Microfilariae (mf) were enumerated by filtration of 1 ml nocturnally collected blood through a Millipore® filter. Plasma, for immunological studies was stored (immediately after separation) at -20°C for several months and subsequently transported to The Netherlands on dry ice, where it was stored at -70°C until use.

Parasite antigen

Adult *B.malayi* worms were purchased from TRS laboratories, Athens, GA, USA. Female worms (100) were freeze-dried, ground to powder on ice, dissolved in PBS (phosphate bufffer saline) and slowly stirred overnight at 4°C to obtain solubilized *B. malayi* antigen (BmA). The protein concentration was determined by the BCA (2,2'-Biquinoline-4,4'-dicarboxylic acid disodium salt hydrate) methods [303] before storage at -20°C.

ELISA for detection of BmA-specific IgE

Microtitre plates (Nunc-Immuno Plate, Maxisorp, Inter med, Denmark) were coated overnight at 4°C with 100 μl/well of BmA (5 μg/ml) in 0.1 M carbonate buffer (pH 9.6) and blocked with 120 ml of PBS containing 5% bovine serum albumin (BSA: Organon, Teknika, Boxtel, the Netherlands). Patient plasma samples, diluted 1/20 in assay buffer (Tris 0.1 M pH 7.5 containing 0.05% Tween-20) were incubated at 100 μl/well. The plates were then incubated at 37°C 15 minutes on a shaker machine incubator and subsequently washed with PBS containing 0.1% Tween-20. This washing step was repeated after each of the following steps. Biotinylated goat anti human IgE antibodies, 100 μl/well were added (Vector laboratories, CA, USA; diluted 1/1000), followed by alkaline phosphatase-conjugated streptavidin (Boehringer Mannheim, Germany; diluted 1:3000). Both conjugates were diluted in 0.1M Tris-HCl containing 0.05% Tween-20 and incubation was performed at 37°C for 15 minutes while shaking. The color was developed for 1.5 hours by addition of *para*-nitrophenylphosphate substrate (*p*-NPP [Boehringer Mannheim, Germany]) diluted in diethanolamine (DEA) buffer (0.5mM MgCl2, 0.1 M DEA, pH 9.6[Merck, Germany]) and optical density (OD) was measured at 405 nm on a Microplate Autoreader (Bio-Tek Instruments). The OD value was converted into international units (IU) of IgE using a standard curve (10 three-fold dilutions) of a serum originating from a patient from Central Sulawesi with known anti-BmA IgE titer (205 IU/ml) as determined by RAST. A cut-off value was determined by taking the mean of BmA-specific IgE titers plus 3 times the standard deviation (SD) using sera of 20 healthy Dutch donors at the Blood Bank in Leiden (2.86 IU/ml).

RAST for detection of BmA-specific IgE

RAST for detection of BmA-specific IgE was done as described previously [73]. Briefly, *B. malayi* adult antigen was coupled on to cyanogen-bromide-activated Sepharose CL-4B (Amersham Pharmacia Biotech, Uppsala, Sweden) at a ratio of 150 μg BmA to 100 mg of Sepharose, further referred to as BmA-Sepharose. The assay for specific IgE was

conducted by incubating 50 µl plasma with 15µg BmA-Sepharose in a final volume of 350 µl PBS containing 0.3% BSA and 0.1% Tween 20 (PBS-AT). After an overnight incubation shaking at room temperature, Sepharose was washed five times with PBS containing 0.1%Tween 20 (PBS-T). Subsequently, the Sepharose was incubated overnight on an end-over-end rotator at room temperature with 50 µl of ^{125}I radiolabeled sheep antibodies directed to human IgE (Sanquin, Amsterdam, The Netherlands). After four washing steps with PBS-T, the samples were counted in a gamma counter. Percentages binding were converted to IU/ml of IgE using a standard curve of chimeric monoclonal IgE antibodies against *Der p 2* and Sepharose-coupled recombinant *Der p 2* [304]. A cut-off was determined by taking the mean IgE titers plus 3 times the SD of 20 healthy Dutch donors at the Blood Bank in Leiden (0.4 IU/ml).

ELISA for detection BmA specific IgG4

The levels of anti-filarial IgG4 were determined in ELISA which has been described elsewhere [305]. The OD of patient plasma were converted into arbitrary units (AU) using a standard curve of a positive donor from Central Sulawesi with 10^6 AU/ml. The mean of BmA-specific IgG4 reactivity plus three times the SD of healthy Dutch donors at the Blood Bank in Leiden were determined as cut-off (15800 AU/ml).

Statistical analysis

Statistical analysis was performed in SPSS for Windows version 10.0. The relationship between two methods was investigated by calculating Pearson's correlation; linear regression analysis was carried out to perform the correspondence and the concordance was calculated using the *chi square* test.

Result

Study subjects

A total of 768 individuals from Kalia and Salubarana aged 5 – 82 years participated in this study as shown in table 1. The number of male and female participants in the two villages was comparable. The prevalence of filarial infection determined by conventional blood filtration technique was slightly higher in Kalia than in Salubarana but this was not statistically significant. The prevalence and the levels of anti-filarial specific IgG4 were similar in the two villages. Therefore, the population were combined in all subsequent analyses.

Comparison of RAST and ELISA to determine anti-filarial IgE

The geometric means (GM) of the BmA specific IgE by ELISA and RAST are 1.9 IU/ml (IQR=0.76-9.20) and 3.09 IU/ml (IQR= 1.40-7.62), respectively. Based on regression analysis, regression equation was formulated as follows:

$$\textbf{ELISA = 4.74 RAST − 11.32}$$

	Village		Total
	Kalia	Salubarana	
Number of subject	302	466	768
Female/male	157/145	225/241	382/386
Mean age	26.16	28.89	27.8
Microfilaria prevalence (%)	9.6	8.6	9
Specific IgG4 prevalence (%)[a]	62.9	62.4	62.6
Mean specific IgG4 level (A.U./ml)[b]	4.42	4.58	4.51
Range specific IgG4 level (A.U./ml) [b]	1.88-6.70	1.90-6.70	1.88-6.70

Table 1. Summary of the study population.
A.U, arbitrary units.
[a]Percentage of individuals with specific IgG4 above the cut-off value.
[b]Mean and range of anti-filarial IgG4 are given as $\log_{10}$ values of arbitrary units (mean ± standard deviation).

Likewise, Pearson's correlation coefficient = 0.69 (p<0.001). From figure 1, it is also clear that at lower levels of specific IgE, the correlation between the two test methods was poor. In several samples that were negative by ELISA, low levels of IgE could be detected by RAST. Indeed, when the cut-off level of each assay was considered, 94.5% of the population was positive by RAST in contrast to only 52.6% by ELISA. As shown in table 2, although an overall significant concordance was found between the two assays (P<0.001), 328 (42.7%) individuals with a positive RAST result were negative in the ELISA, whereas only 6 (0.8%) subjects were positive by ELISA, yet negative by RAST.

Performance of the assays as a function of infection status
When the performance of the two tests, ELISA and RAST was examined as a function of infection status, we found that the correlation coefficient in mf positive and in IgG4 positive individuals was higher than in IgG4 negative subjects (correlation coefficient = 0.91 in mf positives, 0.70 in IgG4 positive and 0.52 in IgG4 negative individuals) (Figure 2).

Performance of the assay in children and adult as a function of gender
When the study population was classified as children (aged≤16 years) and adult (age>16 years), we found that the correlation between the two assays increased with increasing age (correlation coefficient=0.63 and 0.71 in children and adults, respectively; P<0.001). Antibody levels for each age group were then analyzed according to gender. Levels of anti-filarial IgE measured by ELISA did not differ significantly between children and

adults in either females or males (figure 3A). Males had significantly higher IgE levels compared to females (P<0.001). In the RAST assay, however, anti-filarial IgE levels of male adults were significantly higher than those of male children (P<0.001) (figure 3B) but there was no significant different between female adults and children.
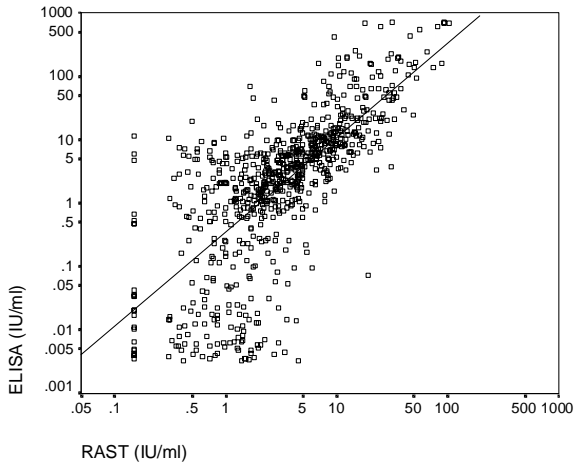

Figure 1. Correlation between BmA-specific IgE measured by ELISA and by RAST. Linear regression is shown as a fitted line.

	Negative for RAST (<0.4 IU/ml)	Positive for RAST (≥0.4 IU/ml)	Total
Negative for ELISA (<2.46 IU/ml)	36	328	364
Positive for ELISA (≥2.46 IU/ml)	6	398	404
Total	42	726	768

Table 2.Concordance between ELISA and RAST for detection of BmA-specific IgE (cut-off values are shown in parenthesis). These values were determined by taking the mean IgE levels (ELISA and RAST) plus three times the SD of 20 healthy Dutch donors at the Blood Bank in Leiden.

Discussion

There is much interest in measuring IgE in immunoepidemiological studies of helminth infections. The interaction of IgE antibodies with FcɛRI and II leads to a number of cellular reactions that may be important in immune clearance of helminths on the one hand and immunopathology on the other. We have compared two widely used immunological methods
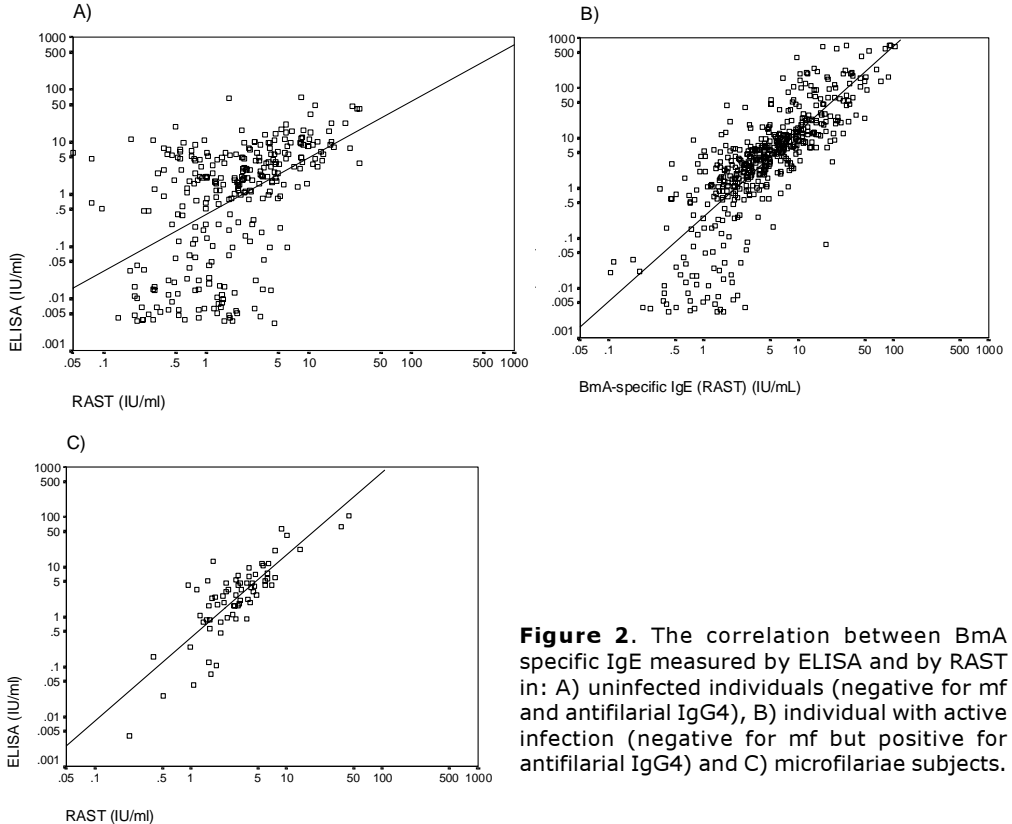

Figure 2. The correlation between BmA specific IgE measured by ELISA and by RAST in: A) uninfected individuals (negative for mf and antifilarial IgG4), B) individual with active infection (negative for mf but positive for antifilarial IgG4) and C) microfilariae subjects.

(ELISA and RAST) to detect IgE, in a large study population residing in an area endemic for brugian filariasis. A highly significant correlation between IgE levels detected by the two methods was found at the population level. The age-prevalence curves or the ratio of IgE/IgG4 analyzed as a function of age, showed similar results in the two tests (results not shown) indicating that the ELISA can also be used to study epidemiological patterns that aim to predict immunity or pathology.

However, the data show that the RAST detected a higher proportion of the population with specific IgE to *B.malayi* compared with ELISA. The better performance of the RAST

is largely attributable to its superior ability to detect low levels of IgE. Indeed, when performance of the test was evaluated in infected (IgG4 positive) and non infected (IgG4 negative) subjects it was clear that the correlation between ELISA and RAST was
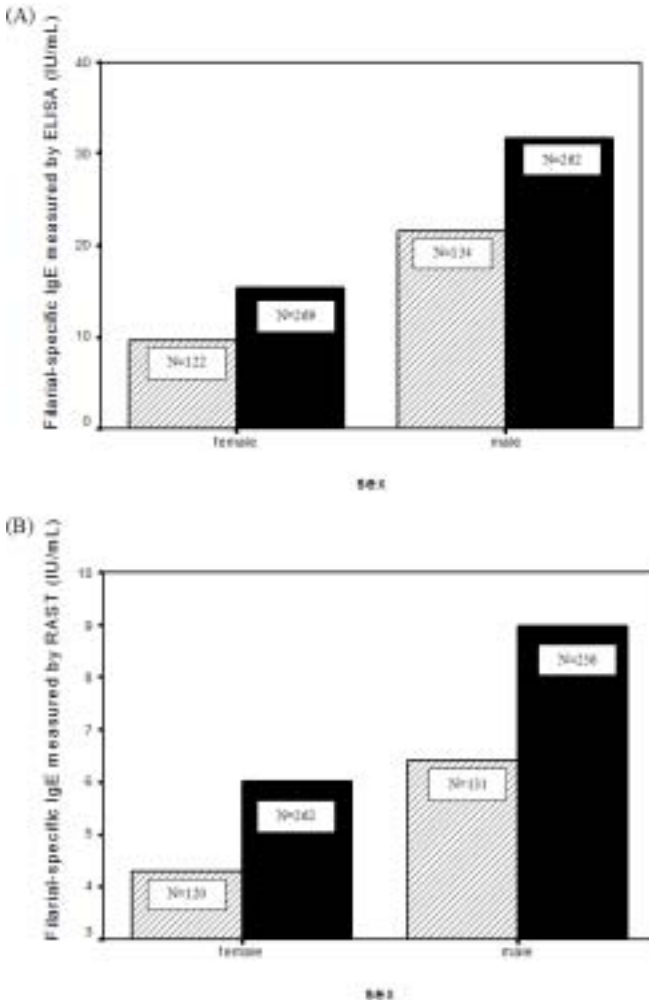

Figure 3. Anti-filarial specific IgE measured by (A) ELISA and (B) RAST of children (d" 16 years; hatched bars) and adults (>16 years; dark bars), in female and male subjects. Data are presented as means ± SE; N=number of subjects in each group.

better in infected subjects as defined by positivity for mf or anti-filarial IgG4 (see Figure 2). This was largely due to higher levels of specific IgE in infected (IgG4 positive) subjects, which allowed a better performance of the ELISA test than in uninfected (IgG4 negative) individuals who often have lower IgE levels.

The ELISA and RAST methods have been compared in few studies of helminth infections. Among patients with hydatidosis, the percentage of positive RAST cases (81.2%) was slightly lower than the percentage of positive ELISAs (90.6%) [301], but in schistosomiasis, the RAST method (detected 72%) was highly superior to ELISA (detected 20%) in detection

and quantization of specific IgE antibodies [302]. Both studies involved small number of subjects. Taking the present study into consideration, the discrepancy between these latter two studies with respect to the performance of ELISA and RAST may be explained by the high levels of IgE in hydatidosis but low levels in schistosomiasis.

In summary, by using sera from a large population, we have shown that there is a strong overall correlation between RAST, a less field applicable method, and the user friendly ELISA. For immunoepidemiological studies it is possible to employ the ELISA method. However, the sensitivity of the ELISA does not allow measurement of low levels of IgE ($<$ 5 IU/ml). The data show that when IgG4 levels are $> 10^4$ AU/ml, it is possible to use the ELISA, but in populations with lower levels of anti-filarial IgG4, the RAST method will have to be used. It follows that in areas where filariasis is highly endemic, one would expect to be able to use the ELISA method whereas in areas with a low intensity of transmission or in areas where control programs are implemented, the RAST method will have to be the method of choice.