A rapid dipstick test for serological diagnosis of brugian filariasis : evaluation results

International collaboration

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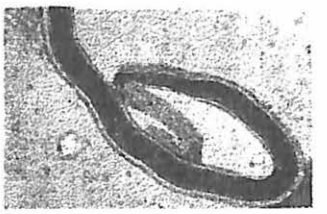
Introduction

Brugia malayi infection is endemic in several Asian countries : Malaysia, India, China Indonesia, Thailand, Vietnam, Philippines



- ~13 million people infected

New test needed for brugian filariasis with following features:

- Specific & sensitive
- Does not need night blood sampling
- Rapid
- Easy to perform and interpret
- Field applicable

B. malayi recombinant antigen (*BmR1)*

⇓

Indirect ELISA

Trans Roy Soc Trop Med Hyg 2001; 95:280-284

⇓

Further purification and concentration

⇓

Development of dipstick test

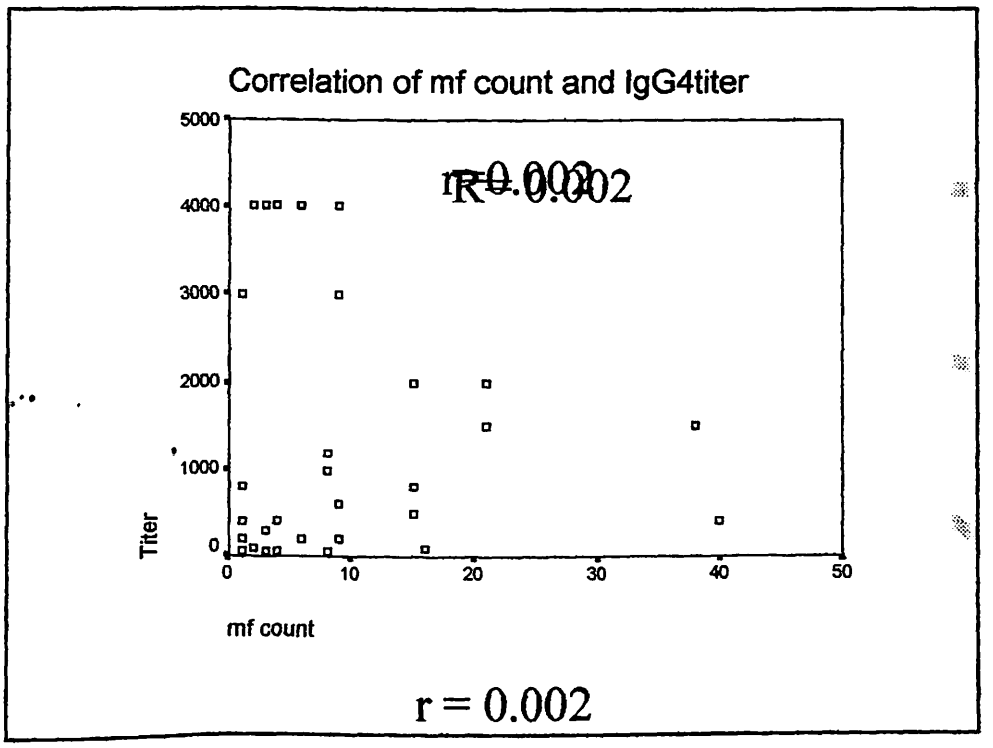
Dipstick test (Brugia Rapid) :

- Detects specific IgG4 antibodies in infected individuals
- Immunochromatography
- 10-15 minutes

Sensitivity

Sensitivity studies

	mf+	Dipstick +ve	Dipstick -ve	Sensi-tivity
India	51	50	1	98.0%
Indon	108	101	7	92.6%
Nether-lands	207	191	16	92.3%
M'sia	69	69	0	100%
Swiss	6	6	0	100%
Total	441	417	24	94.6%



r = 0.002

Specificity

Specificity studies I

Soil-transmitted helminths

	No. samples	Dipstick -ve	Dipstick +ve	Specifi-city
M'sia	51	50	1	98%
Indon	19	19	0	100%
Swiss	6	6	0	100%
Total	76	75	1	98.7%

Specificity studies II

Non-filarial helminths

	No. samples	Dipstick -ve	Dipstick +ve	Speci-ficity
M'sia	50	49	1	98.0%
Indon	10	10	0	100%
Swiss	49	49	0	100%
Total	109	108	1	99.1%

Specificity studies III

Protozoa

	No. samples	Dipstick -ve	Dipstick +ve	Specifi-city
M'sia	225	223	2	99.1%
Indon	13	13	0	100%
Swiss	19	19	0	100%
Total	257	255	2	99.2%

* 12 samples from bacterial & viral infections were all dipstick negative

Specificity studies IV

Healthy people

	No. samples	**Dipstick -ve**	**Dipstick +ve**	**Specifi-city**
M'sia	**130**	**128**	**2**	**98.5%**
Nether-lands	**220**	**220**	**0**	**100%**
Swiss	**10**	**10**	**0**	**100%**
USA	**36**	**36**	**0**	**100%**
India	**20**	**20**	**0**	**100%**
Total	**416**	**414**	**2**	**99.5%**

	Microfilaria positive	Microfilaria negative	Total
Dipstick positive	417	6	423
Dipstick negative	24	864	888
	441	870	1311

Sensitivity **417/441 = 94.6%**

Specificity **864/870 = 99.3%**

PPV **417/423 = 98.6%**

NPV **864/888 = 97.3%**

Other evaluation results

'Endemic normals'

1. Malaysia (low endemicity)

   28 out of 1134 were pos (~2.5%)

2. India (higher endemicity)

   3 out of 30 were positive (10%)

⇒ detects cryptic infections

Field trial in Sarawak, Malaysia

	No. samples	Thick blood smear positive	Dipstick positive
Miri	238	2	8
Kuching	206	5	31
	444	7 * (1.58%)	39 (8.78%)

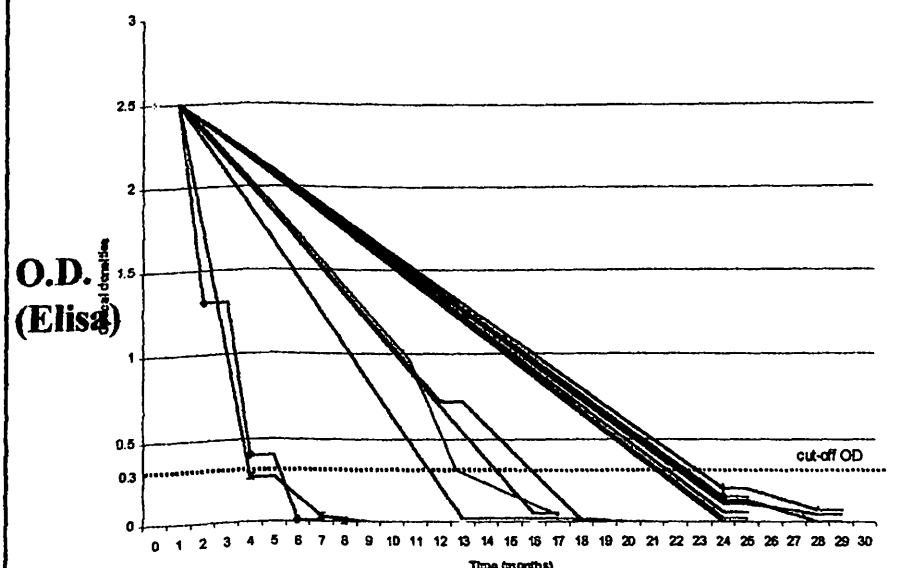
* All dipstick positive

Treatment studies
(i) Treatment of microfilaraemics

	No. samples	Period post-treatment	Dipstick negative
M'sia	15	6 mo - 2 yrs	15 (100%)
Nether-lands	55	1 - 3 yrs	19 (35%)
India	21	1 yr	9 (30%)

Malaysia

Figure 2



Period post-treatment

(2) Treatment of amicrofilaraemics

#	0 mo.	2 mo.	5 mo.	Comments
1	200	100	NA	↓
2	6400	6400	1600	↓
3	800	400	200	↓
4	200	100	50	↓
5	800	400	200	↓
6	400	200	100	↓
7	400	200	NA	↓
8	100	*Negative*		X
9	50	50	*Negative*	X
10	800	400	*Negative*	X
11	400	200	*Negative*	X
12	400	200	*Negative*	X
13	200	200	NA	unchanged

Symptomatic amicrofilaraemics

	No.	Dipstick -ve	Dipstick +ve
Swiss	6 adenolymph.	0	6(100%)
Indon.	17 -adenolymph. -lymphoedema	10(59%)	7 (41%)
Nether-lands	1	1 (100%)	0
Total	24	11 (46%)	13 (54%)

Chronic infections

	No. sample	Dipstick -ve	Dipstick +ve
India	30	19 (63%)	11 (37%)
Indon	43	25 (58%)	18 (42%)
Nether-lands	116	45 (39%)	71 (61%)
Swiss	7	6 (86%)	1 (14%)
Total	196	96 (49%)	101 (51%)

***W. bancrofti* mf+**

	No. samples	Dipstick +ve	Dipstick -ve	Sensiti -vity
USA	56	30	26	53.4%
Indon	20	14	6	70.0%
India	22	12	10	54.5%
Swiss	10	9	1	90%
Total	108	65	43	60.2%

- **Not sensitive for detection of bancroftian filariasis**
- **Cannot be used to differentiate the two filaria species**

Non-lymphatic filaria

	No. samples	Dipstick -ve	Dipstick +ve	Specifi -city
USA	133	124	9	93.0%
M'sia	8	8	0	100%
Swiss	12	12	0	100%
Total	153	144	9 *	94.1%

* 8 Loa-loa, 1 Oncho

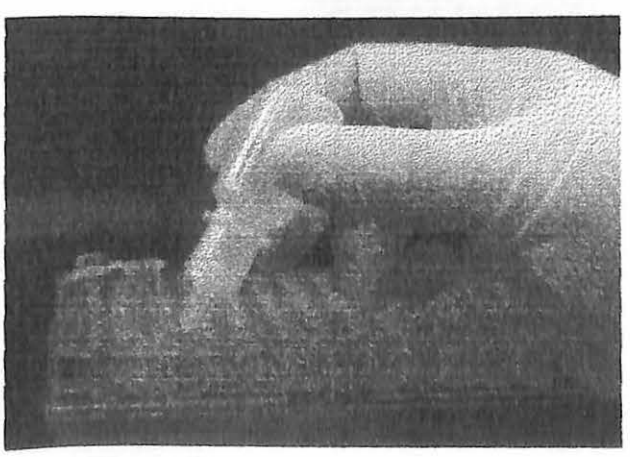
Conclusion

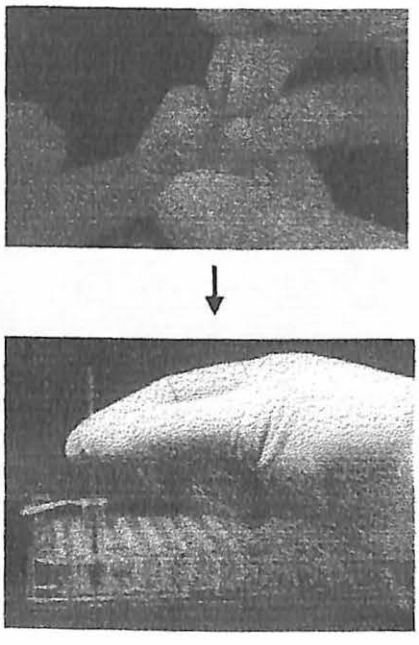
Good specificity & sensitivity, thus is potentially very useful for use in diagnosis and elimination program for brugian filariasis

Brugia Rapid

Dipstick with blood filter

MAb anti-human IgG4-gold

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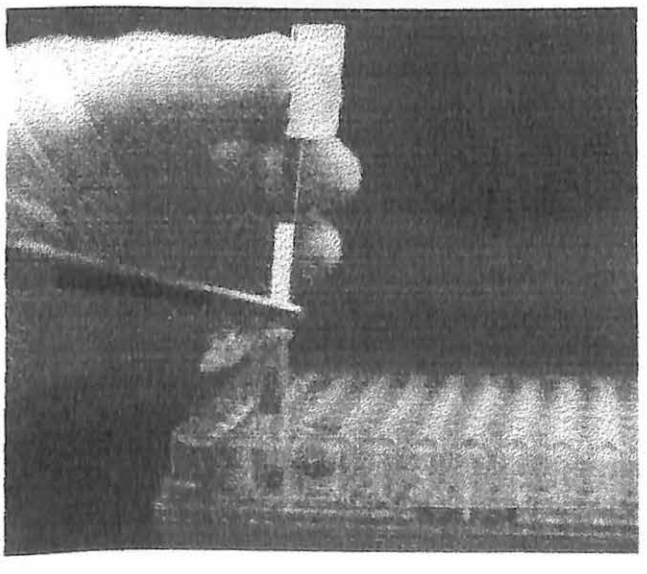
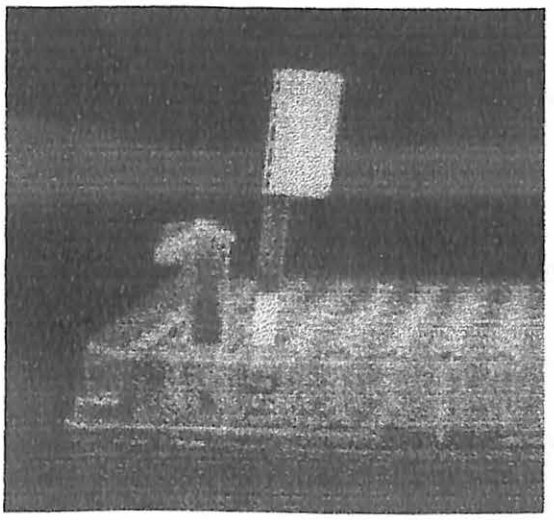
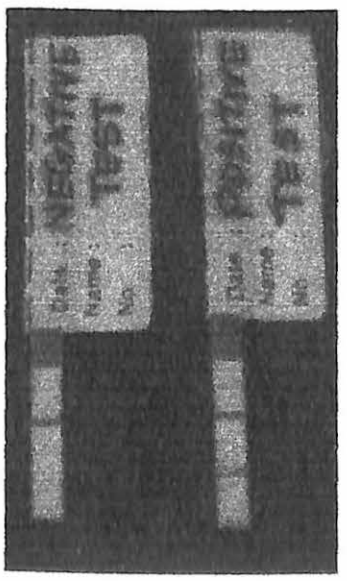




DISCARD



Possible areas for future improvements

1. **Increase simplicity of procedure for greater field applicability.**

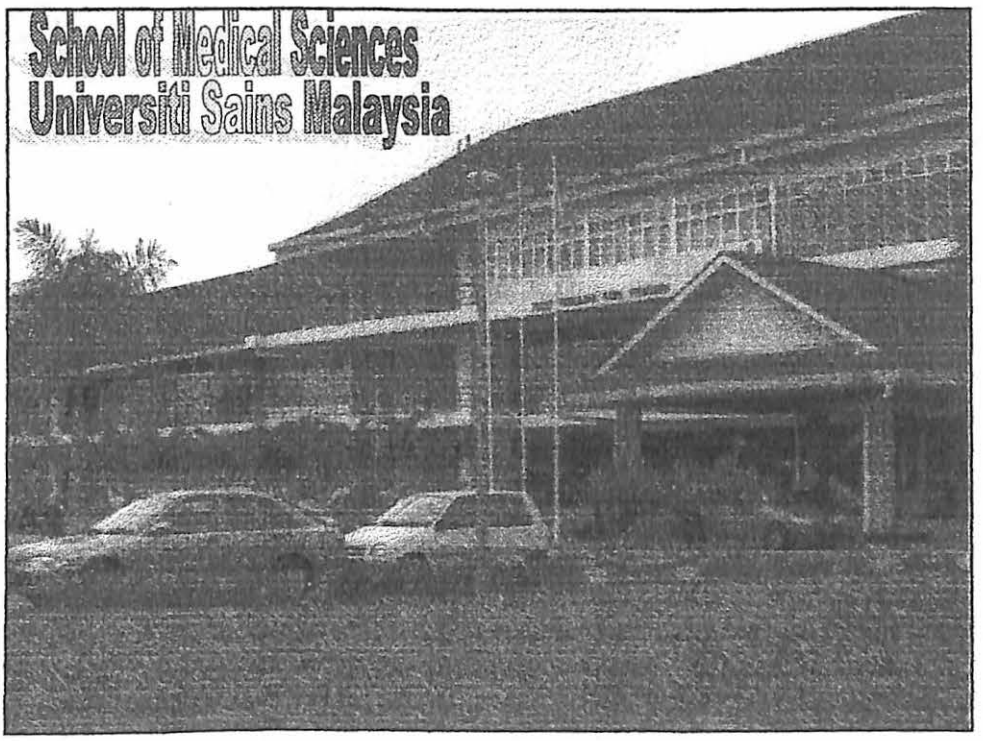
2. **Use of cocktail of recombinant antigens :**

   i. **increase sensitivity of current test**

   ii. **sensitive detection of both brugian and bancroftian filarial infections**

Research funds received from:

- Malaysian Ministry of Science & Technology
- Universiti Sains Malaysia
- UNDP/World Bank/WHO(TDR)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A rapid dipstick test for the serological diagnosis of brugian filariasis: evaluation results

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Abstract

A total of 753 serum samples from six institutions were used to evaluate an immunochromatographic rapid dipstick test (***Brugia Rapid***) for diagnosis of *Brugia malayi* infection. The samples comprised of sera from 207 microfilaria positive individuals and 546 individuals from filaria non-endemic areas. The latter consisted of 70 individuals with soil-transmitted helminthic infections, 68 individuals with other helminthic infections, 238 individuals with protozoan infections, 12 individuals with bacterial and viral infections and 158 healthy individuals. The dipstick is lined with goat anti-mouse antibody (control line) and a *B. malayi* recombinant antigen (test line). First, the dipstick is dipped into a well containing diluted patient sera, thus allowing specific anti-filarial antibody in the serum to react with the recombinant antigen. Then the dipstick is placed into an adjacent well containing reconstituted anti-human IgG4-gold. After 10 minutes, development of two red-purplish lines denotes a positive result and one line indicates a negative reaction. The overall results of the evaluation showed 97% sensitivity, 99% specificity, 97% positive predictive value and 99% negative predictive value. ***Brugia Rapid*** is thus a promising diagnostic tool for detection of *B. malayi* infection, and would be especially useful for the brugian filariasis elimination programme.

Introduction

The World Health Organization has initiated a global programme for the elimination of lymphatic filariasis, namely *Wuchereria bancrofti, Brugia malayi* and *Brugia timori.* (WHO, 1997.) By 2020, this disease which infects approximately 120 million people, could be eliminated. This optimism is based on several reasons, one of them is the availability of a rapid field-applicable diagnostic tool for *W. bancrofti*, the major species of lymphatic filarial parasite. However a similar tool for detection of brugian filariasis is still being sought, since it infects approximately 13 million Asians, mainly in India, Indonesia, South China, Vietnam, Thailand, Philippines and Malaysia.

Previously we have reported on a sensitive and specific recombinant antigen-based Ig4-ELISA for detection of *B. malayi* infection (RAHMAH *et al.*, 2001). This recombinant antigen and immunoassay has now been further developed into a rapid immunochromatographic dipstick test format (***Brugia Rapid***) and an evaluation study performed on this test is hereby presented.

Materials and methods

Serum samples

A total of 753 serum samples were obtained from six institutions of three countries namely 295 samples from Universiti Sains Malaysia (USM), 158 samples from University of Indonesia (FKUI), 50 samples from T.D. Medical College (TDMC, India), 100 samples from Universiti Malaya (UM, Malaysia), 100 samples from Institute of Medical Research (IMR, Malaysia) and 50 samples from Universiti Kebangsaan Malaysia (UKM). All sera used were from the sera bank of each institution, these samples were previously obtained from consenting individuals in accordance with the requirements of each institution. For the USM samples, the following sera were kindly contributed by various researchers : 4 microfilaria positive sera and 9 soil-transmitted helminthiases sera from Dr. Chotechuang Panasoponkul (Thailand); *Anasakis* sera from Professor Akihiko Yano (Japan); and *Schistosoma mansoni* sera from Professor Hassan Mohamed El-Hady (Egypt). Table 1a shows the kinds of samples obtained from each institution and table 1b shows the details of the samples from the other infections.

Recombinant antigen (*BmR1*)

The novel *B. malayi* recombinant antigen was prepared as previously decribed (RAHMAH *et al.*, 2001). Briefly, 1: 100 dilution of an overnight culture of the recombinant bacteria in Luria Bertani broth was subcultured into Terrific broth and incubated at 37^0C in a shaker-incubator until OD_{600} 0.5 was attained. The culture was then induced with 1mM IPTG for 3 h at 30^0C. The bacterial pellet was reconstituted with lysis buffer containing a cocktail of protease inhibitors. The suspension was then sonicated, centrifuged and the supernatant passed through Ni-NTA affinity column.

Rapid dipstick test (*Brugia Rapid*)

This test was developed using the above recombinant antigen and immunochromatography dipstick technology. Membrane card (Millipore, USA) was manually lined with goat anti-mouse antibody (Arista Biologicals, USA) as the top control line and the recombinant antigen as the second test line; these two lines are about 5 mm apart. The lined card was dried and treated with a blocking solution. An absorbent pad (3 cm) was pasted on the adhesive area on top of the card, then the pad was covered with blue vinyl tape. A serum filter (~ 5 mm) was pasted onto the adhesive area at the bottom of the card. The assembled card was then cut into 4 mm strips. Monoclonal anti-human IgG4 antibody (Zymed, USA) was sent for gold-conjugation (Arista Biologicals, USA); a gold solution mixture was prepared and then dried onto plastic wells. In a dry room (20% relative humidity), the tests were placed in individual pouches; each pouch contained a dessicant, a dipstick and one paired wells. The paired wells consisted of one empty well (A) attached to another well (B) containing the dried gold conjugate. The

pouches, dessicants, strip cutter, heat sealer and dry room facilities were kindly provided by a local diagnostic kit manufacturer (Malaysian BioDiagnostic Research).

The test was performed as follows: Upon opening of the pouch, the paired wells are placed snugly into an empty microwell (96-well plate which functions like a rack). 25 µl of phosphate buffered saline (PBS, pH 7.2) is placed in well B and 15 µl of the buffer in well A. Next, 15 µl of serum is added to well A and briefly mixed with the buffer. The dipstick is then placed in well A and the sample is allowed to flow up the nitrocellulose strip by capillary action. In the meantime, the reconstituted reagent in well B is mixed with a pipettor. When the sample front almost reaches the blue vinyl tape, the dipstick is lifted and using a pair of scissors, the serum filter is cut off (and thrown into a container with disinfectant). The dipstick is then placed in well B and the timer set for 10 minutes. The top line, containing goat anti-mouse antibody, serves as a control to ensure the stability of gold-conjugation of the monoclonal anti-human IgG4 antibody, thus it must appear (as red-purplish line) after the completion of either a positive or negative test. Failure of appearance of the control line indicates a defective test. The bottom line is the test line, containing *B. malayi* recombinant antigen, which only develops when the serum sample contains anti-filarial IgG4 antibodies specific to the antigen. Thus the final result in a positive test is the appearance of two red-purplish lines. When a negative serum is used, binding with the recombinant antigen does not occur, thus only the top control line will develop/appear as one red-purplish line.

For USM and Indian samples, the tests were performed at USM. These sera were randomized by a technician from another department and the sample identities decoded after the dipstick results were interpreted. For the other samples, the tests were performed by the personnel of the respective institutions.

Results

Table 2 shows a summary of the results of the evaluation while table 3 shows the statistical analysis of the results. The latter demonstrated 97% sensitivity, 99% specificity, 97% positive predictive value and 99% negative predictive value. The 1 % (6/546) false positive results were obtained from 2 serum samples of blood donors, 2 serum samples from *E. histolytica* infections, 1 serum sample from hookworm infection and 1 serum sample from *Toxocara* infection. The false positives are not restricted to any particular group of infections, and thus may be due to factors other than the respective infections. The 3.4% (7/207) false negative results were obtained from seven microfilaraemic samples from Indonesia. No false negatives were obtained from micrifilaraemic samples from Malaysia, India and Thailand.

In general this test which takes ~12 minutes to complete, is easy to perform and to interpret. Since it is performed on a 96-well microtitre plate, at least four to five tests can be simultaneously performed by one person, thus making it more convenient and time-saving, especially when screening large number of samples.

Discussion

A specific, sensitive, rapid, field-applicable test for detection of *B. malayi* infection is urgently needed for accurate mapping of brugian filariasis endemic areas in the ongoing lymphatic filariasis elimination programme. It would be desirable if a monoclonal antibody is available that can be developed into a good antigen detection rapid test for *B.*

malayi infection, similar to that of the *W. bancrofti* ICT card test. However, until such a test is developed, the best alternative would be the use of recombinant antigen(s) that can detect specific anti-filarial IgG4 antibodies. This antibody subclass has been shown to be elevated in active lymphatic filaria infection (OTTESEN *et al.*, 1985; KWAN-LIM *et al.*, 1990; KURNIAWAN *et al.*, 1993; RAHMAH *et al.*, 1998a; HAARBRINK *et al.*, 1999) and to significantly decline after chemotherapy (WAMAE *et al.*, 1992; Mc CARTHY *et al.*, 1995; KURNIAWAN *et al.*, 1995).

In this study, a newly developed rapid dipstick test ***(Brugia Rapid)*** for detection of *B. malayi* infection was evaluated for sensitivity and specificity using 753 sera samples from six institutions. Statistical analysis of this first evaluation study showed encouraging results i.e. 99% specificity, 97% sensitivity, 99% negative predictive value and 97% positive predictive value.

Among the 207 sera from microfilaraemic individuals; seven samples, all from Indonesia, were found be negative. ***Brugia-Elisa*** using the same recombinant antigen was performed on these seven samples and the results remained negative. However when IgG4-Elisa using soluble *B. malayi* adult antigen was performed, it showed positive results in three (out of seven) of these samples. The other four sera could thus be from individuals who are low IgG4 responders. In a previous study (MARLEY *et al.*, 1995). using *B. pahangi* soluble antigen, 10% of microfilaraemic individuals were found to have low levels of antifilarial IgG4 antibody. A cocktail of two or more highly specific and sensitive recombinant antigens, if available, may further increase the sensitivity and thus the robustness of the dipstick test.

With regards to the sampling areas, five of these false-negative samples were from nocturnal periodic areas of brugian filariasis, while two were from a subperiodic area. In comparison Malaysian samples were from subperiodic areas, while Indian samples were all from nocturnal periodic infections. In order to determine whether strain differences may affect sensitivity of the test, much larger number of samples from each of the periodic and subperiodic areas need to be tested.

Similar to the previous results using the recombinant antigen-based IgG4 ELISA (RAHMAH *et al.*, 2001), initial follow-up studies on treated individuals showed that ***Brugia Rapid*** remained positive for about 6 months to two years post-treatment. More data is being gathered on this aspect; however the possibility of insufficiently treated individuals or of reinfection complicates interpretation of such follow-up studies.

Sensitivity of the dipstick to sera of *Wuchereria bancrofti* microfilaraemic individuals was found to be 54.5 % for samples from India (12/22; samples were kind contribution from Professor P. Kaliraj) and 70% for Indonesian samples (14/20). The reason for the cross-reactivity is not yet understood. However this should not be a problem since the treatment for both species of lymphatic filaria is similar. In fact it may be advantageous if this test could be further developed to detect most cases of bancroftian filariasis.

Further evaluation studies using bigger sample sizes will soon be conducted. These would also comprise samples from non-lymphatic filarial infections; although in general they are not co-endemic with lymphatic filariasis and thus would not pose a problem. The evaluation studies will include a WHO-sponsored three centre laboratory evaluation, field trials in a moderate *B. malayi* endemic area in Sarawak (East Malaysia) and in brugian filariasis endemic areas in Indonesia. The field trials will utilize blood

tests, whereby a blood filter replaces the serum filter on the dipstick and the sample used will be 50 ul of blood with anti-coagulant.

PCR-ELISA assays have been reported for the sensitive detection of *B. malayi* infection (LIZOTTE *et al.*, 1994; RAHMAH *et al.*, 1998 b, FISCHER *et al.*, 2000). Recently, DNA dipstick test for *B. malayi* infection has been developed (KLUBER *et al.*, 2001) which will allow rapid detection of PCR product. The combined usage of blood sample spots on filter paper and DNA dipsticks would make PCR detection of infected individuals more convenient. But, this will still involve sending samples to central laboratories and on-site test interpretation cannot be performed, thus making the PCR-based test not truly field-applicable. Other than the relative higher cost and the possible mix-up of samples that may occur in the multi-step processing of large number of blood spots; PCR-based DNA dipstick may not detect cryptic infections. Nevertheless, comparison between *Brugia Rapid* and DNA dipstick in a field evaluation would be another important study that should be performed. These two tests may in fact, be useful as complementary diagnostic tools in the brugian filariasis elimination programme. The former IgG4 antibody-detection will probably be positive for a longer time post-treatment, while the latter DNA detection test will likely to be less sensitive in detecting amicrofilaraemic infections. These disadvantages of the two tests could be minimized by utilizing a multitest approach in detection of *B. malayi* infection.

In conclusion, this study demonstrated the high specificity and sensitivity of the ***Brugia Rapid*** dipstick test. Therefore, this is a promising tool to be used as a laboratory diagnostic test and to assist in the brugian filariasis elimination programme.

Acknowledgements

This study was funded by the Malaysian Government's National Biotechnology Directorate development grant, No. 0205MBDBCC02 and Medical Innovations and Technology Development Unit of Universiti Sains Malaysia. We would also like to acknowledge the valuable technical contributions of the following people : Rohana Ab. Rahman, Ahmad Dasuki, Khatijah Othman, Dr. Yenny Djuardi, Drs. Heri Wibowo MSc. and Tuan Afifah Tuan Ibrahim.

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Legend to tables

1. Table 1a: Categories of serum samples from various institutions used in the evaluation of ***Brugia Rapid***
2. Table 1b: Details of serum samples from other infections used in the evaluation of ***Brugia Rapid***
3. Table 2: Results of evaluation of ***Brugia Rapid*** using various categories of serum samples from six institutions
4. Table 3 : Specificity and sensitivity of ***Brugia Rapid*** for the diagnosis of brugian filariasis, as assessed by serum samples from six institutions

Table 1 a

Serum samples	**USM**	**FKUI**	**TDMC**	**UM**	**IMR**	**UKM**	**Total**
Microfilaria positive	39	108	30	10	20	0	207
Soil-transmitted helminthiases	39	19	0	0	0	12	70
Other helminth infections	32	10	0	26	0	0	68
Protozoan Infections	72	13	0	47	80	26	238
Other infections	12	0	0	0	0	0	12
Healthy individuals	101	8	20	17	0	12	158
Total	295	158	50	100	100	50	753

Note: Except for the microfilaria positive serum samples, all other samples were from individuals residing in non-filaria endemic areas.

Table 1b

Category	Species	No.	Total
Soil-transmitted helminthiases	*Ascaris lumbricoides*	16	
	Trichuris trichuria	21	
	Hookworm	14	70
	Strongyloides stercoralis	1	
	Mixed infections	18	
Other helminthic infections	*Toxocara*	31	
	Gnathosthoma spinegerum	1	
	Taenia solium (cystercercosis)	11	
	Dirofilaria immitis	5	
	Anasakis	8	
	Schistosoma mansoni	9	68
	Onchocerca volvulus	3	
Protozoan infections	*Toxoplasma gondii*	118	
	Plasmodium vivax & P. falciparum	41	
	Entamoeba histolytica	72	
	Iodamoeba butschlii	2	
	Gardia lamblia	2	238
	Cryptosporidium parvum	2	
	Leishmania	1	
Other infections	*Dengue*	1	
	Scrub typhus	1	
	Hepatitis	4	12
	Salmonella typhi	5	
	Campylobacter jejuni	1	
		Total	388

Table 2

Serum sample	No.	*Brugia Rapid* positive (%)	*Brugia Rapid* negative (%)
Microfilaria pos. individuals	207	200 (96.6%)	7 (3.4%)
Soil-transmitted helminthiases	70	1 (1.4%)	69 (98.6%)
Other helminthic infections	68	1 (1.5%)	67 (98.5%)
Protozoal infections	238	2 (0.8%)	236 (99.2%)
Other infections	12	0 (0%)	12 (100%)
Healthy individuals	158	2 (1.3%)	156 (98.7%)
Total	753		

Table 3

	True positive (mf +)	True negative (mf -)	Total
Brugia Rapid +	200	6	206
Brugia Rapid -	7	540	547
	207	546	753

Sensitivity : 200/207 = 96.6% ~ 97%

Specificity : 540/546 = 98.9% ~ 99 %

PPV : 200/206 = 97.1 % ~97 %

NPV : 540/547 = 98.7% ~ 99%