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Multicentre laboratory evaluation of Brugia Rapid dipstick test for detection of brugian filariasis

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Summary A multicentre evaluation of the Brugia Rapid dipstick test was performed using 1263 serum samples in four international laboratories, i.e. T.D. Medical College (TDMC, India), National Institutes of Health (NIH, USA), Swiss Tropical Institute (STI, Switzerland) and Leiden University Medical Centre (LUMC, Netherlands). In comparison with microscopy, the dipstick demonstrated sensitivities of 97.2% (70 of 72) at TDMC, 91.6% (175 of 191) at LUMC and 100% (six of six) at STI. Sera of chronic patients showed a positivity rate of 11.3% (19 of 168) and 61.2% (71of 116) at TDMC and LUMC, respectively. All 266 sera of non-endemic normals from STI, NIH and LUMC tested negative with the dipstick. At LUMC, sera of 'endemic normals' (amicrofilaraemics with no clinical disease) from an area with approximately 35% microfilaria positivity showed 60.8% positive results (31 of 51), thus demonstrating the likelihood of many cryptic infections occuring in this population. Specificities of the test with *Onchocerca volvulus* sera were 98.8% (80 of 81) and 100% (10 of 10) at the NIH and STI, respectively; while specificity with *Loa loa* sera at the NIH was 84.6% (44 of 52). At the STI, the dipstick test also demonstrated 100% specificity when tested with 75 sera from various protozoan and helminthic infections.

keywords *Brugia malayi*, dipstick test, multicentre evaluation

Introduction

Lymphatic filariasis is targeted by the WHO-initiated global elimination programme (Ottesen 2000). The disease is expected to be eliminated by 2020 and in some countries such as Malaysia, with low endemicities, the target year is much sooner. Accurate mapping of endemic areas is one of the criteria for successful elimination. Thus it is important that a good diagnostic tool be employed in this phase of the programme. For bancroftian filariasis, a rapid antigen detection test is available (ICT Filariasis). Antigen detection assays have not been successfully developed for brugian filariasis, thus detection of filarial-specific IgG4 antibodies provides the next best alternative. This is based on the significantly elevated level of IgG4 antibody in active infection (Ottesen *et al.* 1985; Kwan-Lim *et al.* 1990; Kurniawan *et al.* 1993; Rahmah *et al.* 1998; Haarbrink *et al.* 1999) and its decline post-treatment (Wamae *et al.* 1992; Kurniawan *et al.* 1995; McCarthy *et al.* 1995; Rahmah *et al.* 2001a).

Brugia Rapid is a rapid dipstick test lined with a *Brugia malayi* recombinant antigen. Anti-filarial antibodies in patient sera will react with this antigen, followed by binding of this complex with monoclonal anti-human IgG4 conjugated to colloidal gold. Thus, samples containing anti-filarial IgG4 antibodies that react specifically to the recombinant antigen will result in the appearance of a purple-reddish colour at the test line. Previously, an evaluation study of this test involving six institutions (four in Malaysia, one each in India and Indonesia) showed high sensitivity and specificity (Rahmah *et al.* 2001b). This study is a second validation exercise involving independent evaluations by four international collaborating centres.

Materials and methods

Serum samples

A total of 1263 serum samples from four collaborating centres were employed, namely 240 sera samples from

Table 1 Serum samples employed by the four collaborating centres in the evaluation of Brugia Rapid dipstick test

	TDMC	NIH	STI	LUMC	Total
**Brugia malayi* mf+	72		6	191 (Indonesia)	269
Chronic patients (*Brugia malayi* area)	168			116 (Indonesia)	284
**Amicrofilaraemics from *Brugia malayi* area	–		13	51 (Indonesia)	64
Non-endemic normals	–	36	10	220 (20 Europeans and 200 Malaysians)	266
Treated individuals	–			58	58
Onchocerca volvulus	–	81 (Ghana and Guatemala)	10		91
Loa loa	–	52 (Benin; the only endemic filarial species)	1		53
Mansonella perstans	–		1		1
Wuchereria bancrofti mf+	–	56 (Cook Islands and India)	10		66
Wuchereria bancrofti mf– with elephantiasis and TPE	–	12 (six of each)			12
Sera from Flores, Indonesia	–		25		25
Echinococcus	–		33		33
Schistosoma	–		14		14
Hookworm, *Ascaris*, *Strongyloides*,	–		2, 3 and 1, respectively		6
Diphyllobothrium latum, *Fasciola hepatica*, *Leishmania donovani*, *Sarcocystis*	–		4 (one each)		4
Plasmodium falciparum, *Plasmodium vivax*	–		4 and 1, respectively		5
Entamoeba histolytica, *Giardia lamblia*, *Cyclospora*	–		3, 7 and 2, respectively		12
Total	240	237	150	636	1263

TDMC, TD Medical College; NIH, National Institutes of Health; STI, Swiss Tropical Institute; LUMC, Leiden University Medical Centre.
* Methods used for determination of *Brugia malayi* microfilaraemia status were as follows: TDMC, filtration of 1 ml of blood; LUMC, filtration of 1 ml of blood (Sumatra, Central Sulawesi) or 10 ml of blood (South Sulawesi); STI, examination of 20 μl of blood.

T.D. Medical College, India (TDMC), 237 sera from the National Institutes of Health, USA (NIH), 150 sera from WHO and the STI sera banks at the Swiss Tropical Institute, Switzerland (STI) and 636 samples from the Leiden University Medical Centre, Netherlands (LUMC). These were samples from the sera bank of each institution; they were previously obtained from consenting individuals in accordance with the requirements of each institution. Table 1 describes the samples used at each centre.

Recombinant antigen (BmR1)

Brugia malayi recombinant antigen was prepared as previously described (Rahmah *et al.* 2001a). 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 °C in a shaker-incubator until an OD_{600} of 0.5 was attained. The culture was then induced with 1 mM IPTG for 3 h at 30 °C. 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 produced as previously described with modifications (Rahmah *et al.* 2001b). Briefly, membrane cards (Millipore, USA) were manually lined with the recombinant antigen and goat anti-mouse antibody (Arista Biologicals, USA), with a distance of about 5 mm between the lines. An absorbent pad and a serum filter were attached to the top and bottom of the card followed by placement of blue vinyl tape over the absorbent pad. The assembled card was then cut into 3.8 mm strips. A solution containing monoclonal anti-human IgG4 antibody-gold (Zymed and Arista Biologicals,

USA) was dried onto plastic wells. The tests were placed in individual pouches; each pouch contained a dessicant, a dipstick and one set of paired wells. The paired wells consisted of one empty well (A) attached to another well (B) containing the dried gold conjugate. The paired wells are placed snugly into an empty microwell. A drop of phosphate-buffered saline (PBS, pH 7.2) is placed in each well, A and B. There were two versions of the test, one that used 35 µl buffer to reconstitute the conjugate well (B) and the other used approximately 43 µl buffer (from a dropper bottle). However, the final OD of the gold-conjugate is approximately 3.7 (λ at 540 nm, 40 nm colloidal gold particles) in both versions. Serum is added to well A, with the ratio of buffer to sample being 1:1 followed by placement of the dipstick in the sample well (A). 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 discarded. The dipstick is then placed in well B and the timer set for 15 min. In a positive test, two purple-reddish lines will appear contrary to one in a negative test. Figure 1 shows the appearances of the dipsticks, i.e. unused, positive and negative test results. The dipsticks were couriered to the respective institutions and the evaluations were then performed independently by each collaborating centre.

Results

The sensitivity of Brugia Rapid for brugian infection was compared with microscopy at three centres. At TDMC, the sensitivity was 97.2% (70 of 72); at LUMC 91.6% (175 of 191) and at STI 100% (six of six). Each of the normal, healthy individuals from Europe, USA and Malaysia was negative using the dipstick (100% specificity, 266 of 266). At STI, there was 100% specificity when the dipstick was tested with sera from individuals infected with *Mansonella perstans* (one of one), Echinococcus (33 of 33), Schistosoma (14 of 14), soil-transmitted helminths (six of six), *Plasmodium* (five of five), *Diphyllobothrium latum* (one of one), *Fasciola hepatica* (one of one), *Leishmania donovani* (one of one), *Sarcocystis* (one of one) and intestinal protozoa (12 of 12). With sera from *Onchocerca volvulus* individuals, the NIH recorded a 98.8% (80 of 81) specificity rate while STI recorded no cross-reaction (10 of 10). Specificity with sera from *Loa loa*-infected individuals (obtained from a region where there were no other filariae) was 84.6% (44 of 52) at the NIH, while no cross-reaction was observed at STI (one of one). Evaluations involving sera from *Wuchereria bancrofti* mf+ patients were performed at NIH and STI. At NIH, the dipstick was observed to display 53.6% (30 of 56) cross-reactivity with mf+ sera from the Cook Islands, while mf+ bancroftian sera from Sri Lanka at STI demonstrated 90% (nine of 10) cross-reactivity. No cross-reactivity was observed at NIH when the dipstick was tested with six sera, each of amicrofilaraemics bancroftian patients with elephantiasis and tropical pulmonary eosinophilia (TPE) syndrome. Table 2 shows the statistical analysis using results of the relevant sera samples.

Brugia Rapid dipstick tested with sera of chronic patients, with various grades of lymphoedema from TDMC, gave a low positivity rate of 11.3% (19 of 168) while those from LUMC showed a significantly higher positivity rate of 61.2% (71 of 116). This difference probably reflects the different proportions of active infections in these two populations.

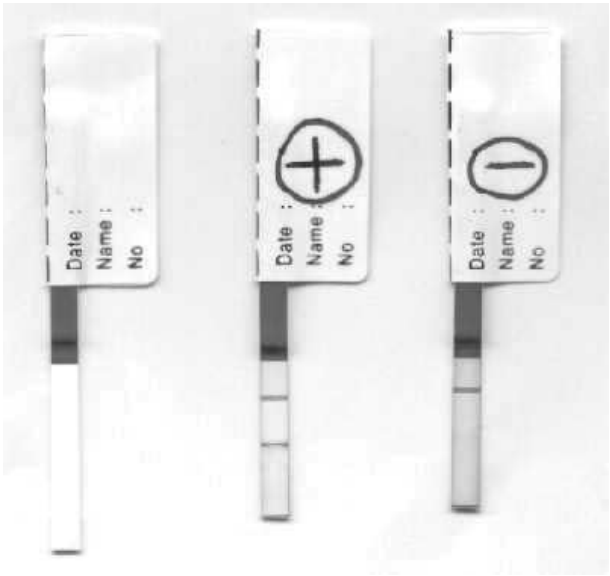

Figure 1 Brugia Rapid dipstick tests. Unused strip (with serum filter), strip with positive result and strip with negative result.

Table 2 Specificity and sensitivity of Brugia Rapid for the diagnosis of brugian filariasis, as assessed by serum samples from LUMC, TDMC, NIH and STI

	True positive (mf+)	True negatives (mf−)*	Total
Brugia Rapid+	251	0	251
Brugia Rapid−	18	266	284
Total	270	266	535

mf, microfilaria.
* True negatives, sera from healthy individuals and those non-endemic area residents with protozoan and helminthic infections, excluding *Wuchereria bancrofti*, *Onchocerca volvulus* and *Loa loa*.
Sensitivity, 251 of 270 = 93.0% (92.96%).
Specificity, 266 of 266 = 100%.

Effect of treatment on response to Brugia Rapid

At LUMC, Brugia Rapid was tested with sera from 58 treated microfilaraemic patients from Central Sulawesi (18 samples), Sumatra (19 samples) and South Sulawesi (21 samples). The treatment regimen for Central Sulawesi and Sumatra comprised one 100 mg tablet per week for 1 year. For South Sulawesi, the regimen was 6 mg/kg DEC for three consecutive 12-day courses, with 2-week intervals between courses during which DEC was administered once a week.

From Central Sulawesi, samples were taken 2 years post-treatment. Fifteen of the 18 individuals were amicrofilaraemic; the dipstick test gave eight (53%) positive and seven (47%) negative results. Three other samples were from individuals who were still microfilaraemic and all were positive with the dipstick test. From Sumatra, all the 19 specimens obtained by post-treatment were from individuals who were amicrofilaraemic. Nine were taken at 1 year post-treatment; seven (78%) of these were dipstick positive and two (22%) dipstick negative. Ten samples were taken 2 years post-treatment; six (60%) were dipstick positive and four (40%) were dipstick negative. From South Sulawesi, serum samples were taken at 3 years post-treatment, at which time all subjects were amicrofilaraemic. Brugia Rapid showed reactivity with 15 (71%) of these sera and negative results with six (29%) samples.

Mf– without clinical disease

At LUMC, the dipstick was also tested with sera from amicrofilaraemic individuals without clinical disease living in areas endemic for brugian filariasis. These individuals came from a population with approximately 35% mf+ rate and filtration of 1 ml of blood was used to determine their microfilaraemia status. The dipstick test showed that 60.8% (31 of 51) of the mf–, disease negative individuals from this population gave positive results.

Mf– with clinical disease

At STI, 53.8% (seven of 13) of sera from amicrofilaraemic individuals with clinical symptoms from Indonesia (Sulawesi and Kalimantan) were positive. Of the seven positive samples, six had adenolymphagitis and one had elephantiasis. Of the six sera that gave negative results, five had elephantiasis/adenolymphangitis and one had elephantiasis alone. In addition the dipstick was also tested with 25 sera from amicrofilaraemic individuals from Flores, Indonesia; five (25%) were positive and one of them had adenolymphangitis. Of the 20 samples from Flores that gave negative results with the dipstick, 10 had adenolymphangitis and 10 had elephantiasis. Flores is endemic for *B. timori*, however co-endemicity with *W. bancrofti* cannot be excluded. However, as microfilaraemia status from samples at STI was based on examination of 20 µl blood, rather than the more reliable method of filtration of 1 ml blood, some of the individuals identified as mf– may be microfilaraemic.

Discussion

The diagnosis of brugian filariasis is still largely dependent on the traditional method of microscopic examination of night blood for the presence of microfilariae. As the lymphatic filariasis elimination programme has already begun, this method has been employed for mapping of the endemic areas prior to mass treatment. Due to the insensitivity of this method, some endemic areas will probably not be included in the programme, which may lead to the possibility of resurgence of infection in these neglected areas. Thus a sensitive, specific and field applicable rapid test would be a very useful tool for the elimination programme. As diethylcarbamazine is an effective microfilaricidal, most infected people will become amicrofilaraemic following the commencement of the annual mass treatment employed in the elimination programme. Until elimination has been achieved, the dependence on microscopy to detect infection in patients from treated populations would be problematic. Thus a serological test would probably be necessary for patient diagnosis in the near future.

The sensitivity and specificity of Brugia Rapid dipstick test for the detection of *B. malayi* infection has previously been reported (Rahmah *et al.* 2001b). This second validation study involved independent evaluations by four collaborating centres. Sensitivity of the dipstick was found to range from 91.6% to 100 %, with a mean sensitivity of 93.0% (Table 2). Of the false negative samples, both sera from India and 50% (eight of 16) of the sera from Indonesia had microfilarial counts which could have been readily detected by thick smear examination. From the previous evaluation study, 85.7% (six of seven) of the false negative samples were from samples with significant levels of microfilaraemia (Rahmah *et al.* 2001b). These false negative cases are most probably due to the low IgG4 antibody responsiveness in these individuals. Approximately 10% of microfilaraemic individuals in a bancroftian filariasis area are low IgG4 responders (Marley *et al.* 1995); the same pattern can be expected from individuals with brugian filariasis. Thus for the diagnosis of patients, we recommend using both Brugia Rapid dipstick test and microscopy to ensure that infected patients who are low IgG4

responders are not missed. Based on this study, if both tests are employed a sensitivity of at least 97% (252 of 262) is achieved for patient diagnosis.

With the exclusion of sera from patients infected with *W. bancrofti*, *O. volvolus* and *L. loa*, the specificity of the test is 100% (Table 2). The latter two filarial species are not co-endemic with *B. malayi*, thus, operationally they need not be included in the determination of the test's specificity. In the previous evaluation study, Brugia Rapid was reported to show 96.6% sensitivity and 98.9% specificity. These values are not much different from the findings in the present study. In order to examine the overall results of the laboratory evaluations of the dipstick thus far, the results of the previous and current studies are combined and presented in Table 3. As shown in this table, the dipstick test displayed an overall sensitivity of 94.7% and a specificity of 99.3%. These high values show that the dipstick test is a good diagnostic tool for detection of brugian filariasis.

In the previous evaluation study, cross-reactivity with mf+ sera from bancroftian patients has been reported to be 54.5% (12 of 22) with sera from India and 70% with sera from Indonesia (14 of 20), thus a mean cross-reactivity of 61.9% (Rahmah *et al.* 2001b). In the present study the cross-reactivity was 53.6% (30 of 56) with mf+ sera from Cook Islands and 90% (nine of 10) with mf+ sera from Sri Lanka, with a mean cross-reactivity of 59% (39 of 66). Further studies are underway to determine the actual extent of cross-reactivity with this related species from different endemic areas. As the endemic areas for brugian and bancroftian filariasis are quite well defined in most parts of Asia, this cross-reactivity is not of a significant concern. In fact, a high degree of cross-reactivity will be beneficial in screening the areas with both lymphatic filarial infections.

Reactivity with sera of chronic patients showed significant variation at the two different centres, i.e. 11.3% (19 of 168) at TDMC and 61.2% at LUMC (71 of 116). This might be explained by the various degrees of chronicity in these patients; indeed, the majority of the grade 1 lymphoedema patients are expected to harbour active infections, while the majority of grade IV lymphoedema patients would be expected to have inactive infections. From the TDMC samples five, 58, 61 and 44 patients had lymphoedema of grades I, II, III and IV, respectively. Thus the low positivity rate of chronic sera from TDMC can be attributed to the fact that most of the sera came from chronic patients of grades III and IV (62.5%, 105 of 168).

Table 3 Specificity and sensitivity of Brugia Rapid for the diagnosis of brugian filariasis, as assessed by the combined results of the previous (Rahmah *et al.* 2001b) and present evaluation studies

	True positive (mf+)	True negatives (mf−)	Total
Brugia Rapid+	451	6	457
Brugia Rapid−	25	806	831
Total	476	812	1288

Sensitivity, 451 of 476 = 94.7%.
Specificity, 806 of 812 = 99.3%.

Preliminary studies on post-treatment positivity of IgG4-Elisa, based on the same recombinant antigen in a low-endemic area, shows that the assay became negative 6 months to about 2 years post-treatment (Rahmah *et al.* 2001a). Post-treatment samples from LUMC showed that the dipstick detected 78% of 1 year post-treatment patients, 53% and 60% of 2 year post-treatment patients, and 71% of 3 year post-treatment patients. The areas from which these post-treatment samples were taken are medium to high endemic areas, the estimated microfilaria prevalence rates were 35% for Central Sulawesi (based on 1 ml of night blood), 42% for South Sulawesi (based on 10 ml of night blood) and 5% for Sumatra (based on 20 µl of night blood). Therefore, interpretation of post-treatment patients is complicated by the possibility of reinfection in these high intensity transmission areas and lack of a gold standard to determine whether all the adult worms are killed by the treatment. Therefore it is important that further data be obtained on the actual extent of positivity of Brugia Rapid post-treatment, especially in the context of the once a year mass treatment in the filariasis elimination programme. This would probably vary among areas of different levels of endemicity. This information would be crucial to determine the suitability of this test as a tool in the phase of certification for disease elimination. Furthermore a test to indicate whether an individual is truly free of infection would be a valuable tool in studying the immunological basis for protective immunity in lymphatic filariasis (Kreiser & Nutman 2002) and for vaccine development.

Disease negative individuals living in filarial endemic areas who are amicrofilaraemic after filtration of 1 ml blood are often termed 'endemic normals', although a proportion are known to have cryptic infections. At LUMC, 60.8% of the amicrofilaraemic, disease negative individuals were found to be positive by the dipstick. This is not surprising as these individuals live in an endemic area with approximately 35% microfilaraemia rate. In a recently completed field trial in Sarawak, 0.9% (23 of 2545) of the population under study was microfilaremic; and 8.5% (215 of 2522) of the individuals who were mf negative by examination of 60 µl blood were positive with the dipstick (M. Jamail, unpublished data). Therefore, the dipstick provides a very useful tool in detection of infected

amicrofilaraemic individuals, particularly in low endemic areas.

Two evaluation studies have shown the Brugia Rapid dipstick test to be highly sensitive and specific for detection of brugian filariasis. It would be very useful for patient diagnosis, screening of grey areas in the mapping activity of the filariasis elimination programme and screening of foreign workers from endemic countries. The test for samples of whole blood/serum/plasma is now being commercialized by Malaysian Bio-Diagnostics Research, thus it is available for further studies and validation by other investigators.

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