

EVALUATION OF THE Og34C FILTER PAPER TECHNIQUE IN LYMPHATIC FILARIASIS PREVALENCE STUDIES

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ABSTRACT

Currently there are several tests which can be used for monitoring and evaluating lymphatic filariasis prevalence in mass drug administration (MDA) programs with the ICT as most commonly accepted field test. However, other techniques to determine circulating antigen are available that also could be suitable in these programs. Our aim was to determine the suitability of the Og4C3 filter paper technique to determine antigen prevalence for lymphatic filariasis. We compared multiple antigen methods during the course of baseline clinical prevalence studies in Papua, New Guinea in over 800 subjects. We found that the ICT and Og4C3 filter paper techniques and using blood from the ICT card in the Og4C3 ELISA were equivalent in performance and that the serum Og4C3 ELISA test detected significantly more antigenic individuals. In addition, we found that modification of the Og4C3 assay by removing the boiling step did not affect its performance. Our results indicate that the Og4C3 filter paper technique is suitable for use in elimination of transmission of lymphatic filariasis monitoring and evaluation programs, quality control of ICT testing could be accomplished by using blood from the ICT test card, and with appropriate logistics in place, venous sampling and testing by the serum Og4C3 ELISA is achievable in monitoring and evaluation and would better

identify areas with low level antigenaemia prevalence and possible ongoing transmission.

Keywords: lymphatic filariasis, diagnostic testing, filter paper collection, quality control, Og34C, ICT

Lymphatic filariasis related morbidity includes both acute inflammatory episodes and chronic manifestations including lymphoedema exacting physical and psychological burdens (1). Lymphatic filariasis was acknowledged to be potentially eradicable by the WHO and member states were urged to develop national plans for its elimination (2). By the end of 2012, 59 countries had completed mapping of foci, 13 countries were in progress, and one country was yet to start. In 2012, 425 million people had received treatment representing coverage of 76% (3).

Historically, lymphatic filariasis infection has been determined by the detection of microfilariae in blood samples by microscopy. However, microscopy is relatively difficult to perform well and consistently in the field (4). Additionally, for many subspecies of *W. bancrofti* the optimum time for sampling is between 10 pm and 2 am when the concentration of microfilariae is greatest in the peripheral blood, therefore requiring the need for night blood surveys. These create problems for surveyed populations and survey staff.

Two commercial antigen tests for lymphatic filariasis are the ICT and the

Og4C3 ELISA. The ICT was chosen in 2000 for mapping lymphatic filariasis (5). A 200-kDa antigen released by *Dirofilaria immitis* and *W. bancrofti* binds two monoclonal antibodies AD 12.1 and DH6.5 (epitope AD-DH). The monoclonal antibody AD 12.1 is used in the ICT (4,6). In *D. immitis*, binding of the antibodies is strongest in the cuticle of both sexes and the epitope is present in many filarial and non-filarial nematodes. However, it is only present in the sera of individuals infected with *W. bancrofti* and has not been shown to be present in sera from patients infected with *B. malayi*, *L. loa*, *Ascaris lumbricoides*, *Strongyloides stercoralis* or *Necator americanus* (7,8).

The ICT uses polyclonal antifilarial antibodies coupled to colloidal gold in solution and a monoclonal antibody bound to a nitrocellulose strip. If filarial antigen is present in a sample, it binds firstly to the colloidal gold bound antibody that then moves along the nitrocellulose strip. The secondary antibody captures this complex on the strip forming a visible line (4). The test was originally developed by ICT diagnostics (Australia) but is now marketed as BinaxNOW Filariasis test in the United States by Alere Scarborough (9). Although the ICT is simple to use, it has a short shelf life, is relatively expensive, and has the appearance of false test lines after 20 minutes (6), which are drawbacks for use.

The Og4C3 ELISA detects the Og4C3 antigen and was produced by TropBio Pty, Ltd, James Cook University (Townsville, Australia), but since June 2013 has relocated to Cellabs (Sydney, Australia). The monoclonal antibody that detects this antigen was produced from stimulating mice with homogenized male *Onchocerca gibsoni*. This antibody binds to a range of filarial and non-filarial nematodes including *O. volvulus*, *D. immitis*, *Ancylostoma caninum*, and *Toxocara canis*. It binds to antigens greater than 130-kDa and between 50-60 kDa. These antigens are constitutional antigens located at the junction of the cuticle and hypodermis, in the

intestinal cells of adults and in intra and extrauterine *O. gibsoni* microfilariae (10). Only a small amount of antigen can be extracted from microfilariae (28 AU/1000 mf) (11).

The filter paper technique refers to the collection of capillary blood onto filter paper and then testing this blood for the constituent of interest. Reasonable correlation has been reported between the sample collected by this technique and those collected by venous sampling (12-15). It has also been used successfully to determine post-treatment filariasis infection rates (16-18). However, Gyapong (18) reported a sensitivity of 50.3% for the Og4C3 filter method compared to microscopy identified microfilaraemia (20 µl thick blood smear; TBS assay). This was not attributed to storage conditions or errors reading the TBS nor due to antigenic differences in *W. bancrofti* between Ghana and Papua New Guinea (PNG). It was considered that possibly the cut-off point was set too high or that 15 µl of whole blood was not sufficient to detect antigen reliably. Gyapong noted that other investigators had also experienced low sensitivity with this method. The Og4C3 filter paper technique is cumbersome as it involves detaching the filter ears and placing them into a tube for further processing.

METHODS

As part of the baseline clinical survey for the PNG Filariasis Elimination Program, venous blood was collected from self-selecting residents into EDTA and SST II BD Vacutainers between 19:00 and 01:00 hours. The self-selecting residents, aged five years or older were from the PNG provinces of New Ireland and West New Britain in 2006.

One hundred microliters of blood was extracted from the SST II tube using a heparinized capillary tube and an ICT card test (Binax NOW Filariasis Test) was performed. The next morning whole blood from the EDTA sample was added to three ears of the filter paper, supplied with the Trop-Ag *W. bancrofti* test kit (Og4C3,

Townsville, Australia) and dried. A 60 μ l thick smear was also prepared from the EDTA tube. The SST II tubes were centrifuged 1-3 days post-collection then stored at 4°C until analysis. The thick films were stained with 3% Giemsa for 30 minutes and examined for microfilariae.

The blood samples were transported to Townsville, Australia and stored at 4°C for up to two weeks before analysis. One hundred microliters of serum was diluted with 300 μ l of diluent, placed in a boiling water bath for five minutes then centrifuged at 2000 g for 15 minutes for the ELISA test. In addition, a comparison between untreated (not boiled) serum samples and treated samples was performed.

The blood soaked filter papers were transferred back to Townsville, Australia for further analysis. The three ears of the filter paper were cut in half and all ears placed in a microtiter tube. Two hundred microliters of diluent was added to paper, the tubes boiled for five minutes, centrifuged at 2000 g for 15 minutes and supernatant then used in the Og4C3 ELISA assay.

The ICT card was opened and the blood soaked filter paper removed, placed into a microtiter tube with 400 μ l of diluent, boiled and the supernatant then tested.

All materials are supplied in the Trop-Ag *W. bancrofti* ELISA kit. Polystyrene plates are supplied pre-coated with Og4C3 monoclonal antibody. Fifty microliters of serum or supernatant was added to a well. Seven standards ranging in concentration from <10 to 32,000 antigen units (AU) were added in duplicate to each plate. Plates were incubated overnight at room temperature then washed three times with the supplied wash buffer. Wells were blocked by adding 50 μ l of hydrogen peroxide and incubating at room temperature for ten minutes followed by three more washes in the wash buffer. Rabbit anti-onchocerca antibody was added and plates incubated for one hour followed by another three washes. Anti-rabbit horse radish peroxidase conjugate was added with

another one hour incubation followed by three washes. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was added and then incubated for one hour. These were read with a spectrophotometer with dual wavelengths at 405 and 492 nm.

The absorbance value of the sample was then compared to the average absorbance values of seven standards run in duplicate to allocate the sample to a titer group. Samples with more than 128 AU (titer group 3) were classified as positive. Although there are seven standards supplied with the kit there was often no discrimination based on absorbance between the high concentration standards, six and seven.

Results were entered into Microsoft Excel (2003) and analyzed using SPSS version 13 (SPSS Inc. Chicago, IL).

Treated and untreated serum specimens were also compared using samples collected from West New Britain, PNG. Treated specimens were treated as in the standard procedure by diluting 1:3 with diluent, boiled for five minutes then centrifuged. Fifty microliters of supernatant was then added to a coated ELISA plate. For the untreated comparison, 50 μ l of serum was added to the ELISA plate. Both treated and untreated specimens were tested in duplicate and both treated and untreated samples from the same individual were placed in the same ELISA plate.

RESULTS

The prevalence for each technique is shown in Table 1. Not all individuals were tested by all techniques due to the difficulty in obtaining venous blood samples and errors in processing. There was a significant difference ($\chi^2=65.1$, df=2, $p<0.001$) between the prevalence obtained by the various techniques. There was no difference between the prevalence obtained by ICT and the filter paper Og4C3 ELISA ($\chi^2=0.18$, df=1, $p=0.67$) while the serum Og4C3 ELISA showed a significantly greater prevalence compared

TABLE 1
Comparison of Antigen Diagnostic Tests

Technique	Antigen Prevalence	Sensitivity compared to Og4C3 ELISA	Specificity compared to Og4C3 ELISA	Positive Predictive value	Negative Predictive Value	Sensitivity in comparison with TBS
ICT	31.5% (n=863) CI: 28.4–34.6	63.6% (n=393)	97.6% (n=423) CI: 95.7–98.9	96.2% CI: 93.0–98.1	74.3% CI: 70.4–77.9	88.6% (n=132) CI: 82.0–93.5
Og4C3 filter paper	32.5% (n=766) CI: 29.2–35.8	67.2% (n=354) CI: 62.1–72.1	99.2% (n=391) CI: 97.8–99.8	98.8% CI: 96.4–99.7	77.0% CI: 70.4–77.9	88.4% (n=121) CI: 81.4–93.5
Og4C3 using filter paper from the ICT		83.2% (n=107) CI: 74.7–89.7	100.0% (n=147) CI: 98.0–100.0	100.0% CI: 96.7–100.0	89.1% CI: 83.3–93.4	93.2% (n=44) CI: 81.3–98.6
Og4C3 ELISA	48.2% (n=817) CI: 44.8–51.6					96.9% (n=128) CI: 92.2–99.1

with the ICT ($\chi^2=48.9$, df=1, $p<0.001$) and the filter paper technique ($\chi^2=41.0$, df=1, $p<0.001$). The prevalence of microfilaraemia by TBS was 16.3% (n=808).

Table 1 also shows the sensitivity and specificity compared to the Og4C3 ELISA and sensitivity compared to TBS. There was no difference in the sensitivity or specificity, using a cut-off of 128 AU/ml, compared to the Og4C3 ELISA between the two techniques. Sensitivity, compared to TBS, ranged from 88.6–96.9%. The serum Og4C3 ELISA had the highest sensitivity of the four techniques.

There were 54 samples within titer group three of the Og4C3 ELISA but only two of these were ICT positive, both of which were amicrofilaraemic. Reducing the cut-off for the filter test from 128 AU/ml to 32 AU/ml increased the sensitivity from 67.2% to 91.5%

(CI, 88.1–94.2). However, the specificity (n=389) decreased to 84.8% (CI, 80.9–88.3). Sensitivity, using a cut-off of 32 AU/ml, compared to microfilaraemia (n=121) was 95.0% (CI, 89.5–98.2).

There were 252 specimens tested by all four techniques. The proportion of positive specimens of this sample was 42.5% (CI: 36.5–48.6), 35.3% (CI: 29.7–41.4), 32.9% (CI: 27.4–39.0) and 32.1% (CI: 26.7–38.1), for the serum Og4C3, Og4C3 using filter paper from the ICT, Og4C3 filter paper and ICT, respectively. For these samples, the sensitivity for the ICT, Og4C3 filter paper and the Og4C3 using filter paper from the ICT was 74.8% (CI: 65.5–82.7), 77.6% (CI: 68.5–81.7) and 83.2% (CI: 74.7–89.7) compared to the standard Og4C3 ELISA test. Of these specimens, 44 were microfilaraemic. The

TABLE 2
Positive-to-Positive Concordance of Tests

		Comparison test Numerator			
Comparison test denominator	Og4C3 ELISA	ICT	Og4C3 Filter paper	Og4C3 using filter paper from ICT	TBS
Og4C3 ELISA		250/393 (63.6%)	238/354 (67.2%)	89/107 (83.2%)	124/369 (33.6%)
ICT	250/260 (96.2%)		207/242 (85.5%)	80/81 (98.8%)	117/254 (46.1%)
Og4C3 Filter paper	238/239 (99.6%)	207/246 (84.2%)		81/85 (95.3%)	107/241 (44.4%)
Og4C3 using filter paper from ICT	89/89 (100%)	80/89 (89.9%)	81/89 (91.0%)		41/88 (46.6%)
TBS	124/128 (96.9%)	117/132 (88.6%)	107/121 (88.4%)	41/44 (93.2%)	

sensitivity of the Og4C3 using the filter paper from the ICT was 93.2% (CI, 81.3-98.6), ICT 93.2% (CI, 81.3-98.6), Og4C3 filter paper 90.9% (CI, 78.3-97.5) and Og4C3 ELISA 97.7% (CI, 88.0-99.9). The specificity of the Og4C3 ELISA using the filter paper from the ICT ($n=147$) was 100% (CI: 97.5-100).

Table 2 shows the positive to positive concordance of the various methods. The Og4C3 ELISA showed high agreement with the other methods with all positive cases by the Og4C3 ELISA using the ICT filter paper identified as positive by the Og4C3 ELISA serum test. There was only one discordant sample positive by the filter paper technique but negative by the standard assay. There were 116 samples negative by the filter paper but positive by the Og4C3 ELISA assay. There was a significant correlation between the two techniques ($r=0.79$, $p<0.001$) with a slope of 1.31 (CI, 1.24-1.39).

Two hundred and fifty boiled supernatants and untreated serum specimens collected from West New Britain were compared in the Og4C3 assay. The absorbance readings between the two measurements were significantly correlated ($r=0.97$, $p<0.001$). A plot of the absorbance readings is shown in Fig. 1. There was a significant ($p<0.001$) difference of the paired mean difference at 80 absorbance units (CI, 52-108) between

untreated and treated OD (untreated OD > treated OD). The positive percentage agreement was 94.6% (CI: 88.7-98.0) and negative percentage agreement 97.1% (CI: 92.8-99.2).

DISCUSSION

Currently, there is only one accepted field test used for the mapping of lymphatic filariasis: the ICT. This test only uses 100 μ l of capillary blood, gives a result in 10 minutes, and can be performed with minimal training. It is useful for initial mapping and is more sensitive than the TBS. However, the ICT has a short shelf life, is relatively expensive, can have false results after 20 minutes. More importantly, there have been problems with the manufacturer supplying cards leading to the PNG Filariasis Elimination Program using the Og4C3 ELISA as the standard test for baseline surveys.

The Og4C3 filter paper technique showed significantly lower antigen prevalence than the serum version. It might be expected that the filter paper technique would have a lower sensitivity compared to the serum version due to a dilution effect when eluting the blood from the paper. The Og4C3 filter paper test uses three ears with approximately 10 μ l of blood per ear and therefore approximately

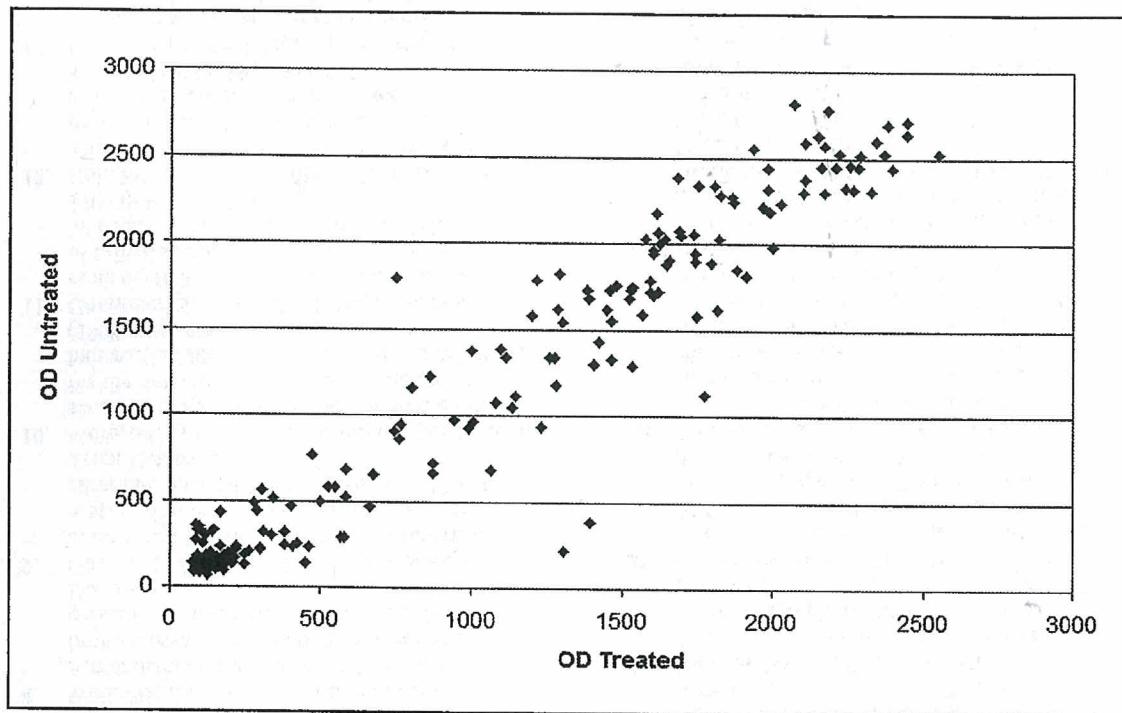


Fig. 1. Graph of OD values in treated versus untreated serum specimens using the Og4C3 ELISA.

12 µl of serum. This is diluted in 200 µl of diluent which is a 4.4 fold reduction in serum concentration tested compared to the Og4C3 ELISA and equivalent to the difference of one titer group. Indeed, a tenfold lower geometric mean titer has been reported (19) and a ROC analysis indicates a cut off of 32 IU/l as the most suitable (9).

During the course of the evaluation, it was observed that the blood soaked filter paper from the ICT card might be able to be used to recheck the ICT result. The ICT, Og4C3 filter paper technique, and the filter paper from the ICT were compared to the serum Og4C3 and TBS. The sensitivity of the all techniques compared to the serum Og4C3 ELISA was poor, but was better when compared to the TBS. The sensitivity of the Og4C3 filter paper test (84%, 81.4-93.5%) compared to TBS was similar to that reported by Hoti et al (19) although lower than the 100% sensitivity reported elsewhere (12-15).

This might be partly explained by the lower cut-off value used in several studies or the difference in techniques determining microfilaraemia. The positive concordance of the ICT and Og4C3 filter paper test was approximately 85%. This compared to 50-60% in a multi-center trial which noted that the majority of tests evaluated did not perform as expected with regard to accuracy and reliability (9).

Testing of the filter pad from the ICT card was shown to be comparable to the Og4C3 filter paper test and with the ICT test itself. It had a significantly higher sensitivity than the Og4C3 filter paper test when compared to the serum Og4C3 which may be due to the greater amount of blood collected on the filter paper pad. Of the 27 specimens from New Ireland that were tested by the Og4C3 ELISA using the filter paper from the ICT and that were positive by the serum Og4C3 ELISA but negative by the ICT, 33%

(n=9) were positive. Testing of eluted blood from the ICT could be an additional quality control procedure when antigen prevalence and concentrations become low after multiple MDA.

The good performance of the assay in the field using precipitate raised the question whether the boiling step was necessary. Serum in the standard Og4C3 assay is heat-treated using the method of Weil and Liftis (7) where one part serum is boiled with three parts sodium EDTA for five minutes. This was to release antigen from immune complexes and to inactivate rheumatoid factor. More and Copeland (10) noted that the sensitivity of the assay was improved by heat pre-treatment and was able to detect less than three antigen units from a minimum detectable antigen level of 256 AU/ml. However, they also noted that this level of sensitivity was not needed since large amounts of antigen were present in most cases and modification for field use would not adversely affect final results. We found this was shown to be correct with 40.8% (102/250) positive by the standard method using boiled supernatant and 40.0% (100/250) using untreated serum. The untreated specimens showed a higher average absorbance compared to their matched treated sample. Although boiling with EDTA appears to release more antigen, this effect is nullified by the dilution effect. The pre-treatment also introduces an extra level of complexity to the procedure that precluded the assay from being introduced into the field.

The requirements for a field test for mass testing are that it uses whole capillary blood, is easy to perform, gives rapid results, is relatively inexpensive, and has good sensitivity and specificity. The ICT meets those requirements although there appears to be significant lot-to-lot variation in sensitivity and specificity. The ICT also has an advantage in baseline surveys where immediate results inform the Elimination Program about its suitability for a sentinel site.

A capillary finger prick sample is

considered more suitable for mass screening compared to venous collection. It is less invasive, and the collection technique easily learned. However, venous night blood collections can occur more efficiently as venous blood can be collected more quickly than capillary blood and the requirement of testing and reading cards at 10 minutes is eliminated. The extra cost of collection materials such as collection tubes, storage tubes and needles is offset by the reduced cost of the Og4C3 ELISA test. Although a rapid test is useful for quickly identifying a sentinel site, the actual base line survey and additional surveys during the elimination program can quite easily be conducted by venous blood collection and the Og4C3 ELISA if there is efficient transportation available to the testing laboratory.

Prevalence by antigenaemia by the Og4C3 ELISA was over 50% greater than the ICT and the filter paper Og4C3 ELISA. A greater prevalence by Og4C3 ELISA has been reported elsewhere where the ICT has also been used (20-23). This could have implications when deciding to stop MDA where continuing low level transmission could be missed. A new version of the ICT has been developed that detects two to four times lower antigen concentration and in trials has recorded higher antigenaemia prevalence levels compared to the older version (24). If consequently shown to be comparable to the serum Og4C3 and suitable for use this is a welcome development. However, the Og4C3 assay would still be useful as a duplicate method for ensuring reliability and testing accuracy within countries' elimination programs.

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