REVIEW ARTICLE

Laboratory diagnosis of lymphatic filariasis in Australia: available laboratory diagnostic tools and interpretation

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Abstract

Lymphatic filariasis (LF) is the second most common mosquito-borne parasitic disease globally and a leading cause of disability. Although LF is not endemic in Australia, individuals returning or emigrating from endemic areas can potentially be exposed to or infected with the filarial nematodes that cause LF, the most prevalent being *Wuchereria bancrofti*. Correct diagnosis requires an understanding of the parasite lifecycle, differences in distribution of the filarial species, and use of the most appropriate diagnostic tests. Since the physician typically does not specify the laboratory tests required to diagnose LF, laboratory scientists are usually responsible for choosing the most appropriate laboratory tests, and for their interpretation. Since there are a number of tests available to diagnose LF, and the choice of test is based on a number of factors including stage of the disease and the region visited, this review aims to clarify the likely tests which should be performed for diagnosis of LF in Australia.

Keywords: Lymphatic filariasis, diagnosis, ELISA, microfilaraemia, antigen, antibody

Introduction

Lymphatic filariasis (LF) is the second most common mosquito-transmitted parasitic disease after malaria, and is caused by the filarial nematodes *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* (Ottesen 2006). Bancroftian filariasis, due to the parasite *W. bancrofti*, occurs throughout the tropics and some sub-tropical regions, and constitutes the majority (90%) of filariasis cases. *B. malayi* is responsible for about 8% of cases and is confined to Asia. *B. timori* causes the lowest proportion of LF cases (2%), and is found in Timor Leste and the Flores islands of the Indonesian Archipelago (Sasa 1976) (Fig. 1). LF continues to be a major cause of morbidity worldwide, with currently 128 million people estimated to be infected and a further 1.3 billion people at risk (WHO 2011).

LF has been eliminated from Australia through improved sanitation, vector control, and the cessation of large-scale importation of labour from LF-endemic areas of the Western Pacific with the last endemic case reported in 1951 (Boreham and Marks 1986). However LF remains an important issue. In 2009-2010 according to the Australian Department of Immigration and Citizenship, there were a total of 208,921 permanent migrations, and, as of 31st December 2010 there were 1,039,687 temporary overseas visitors to Australia (Citizenship 2011). Of this total at 1,248,608, 34% came from LF endemic regions, and thus were possibly infected. This potentially represents 419,989 "endemic" LF patients in Australia. In this paper "endemic" will refer to people who have been born and lived in countries where LF is endemic and "non-endemic" to those not born or living in countries endemic for LF, for example Australia. Secondly, Australian travellers visiting endemic regions may be exposed or infected whilst travelling.

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This debilitating disease is easily treatable in the early stages of infection. However, misdiagnosis or delayed diagnosis will likely have a significant impact on health as the late sequelae of infection can be irreversible (Kerketta *et al* 2005). Consequently, correct diagnosis is imperative, and relies on clinician awareness and correct diagnostic methods. The diagnostic method of choice and sample collection time varies depending on a number of factors. These can include, but are not limited to, the length of stay in an endemic country because of the pre-patent period of the parasite, the endemic region the patient has come from or has visited because of the different available diagnostic tools for the different species and subspecies, and whether the patient is considered to be "endemic" or "non-endemic" (Sasa *et al* 1976; Bennuru and Nutman 2009b). In this review article the available diagnostic methods for LF and their appropriateness for different circumstances are described. Sample collection, diagnostic test performance and test interpretation are discussed.

Laboratory diagnosis

Three laboratory approaches can be used to diagnose LF: parasitological identification of microfilariae (Mf), detection of antigen, and/or detection of antibody. The diagnostic

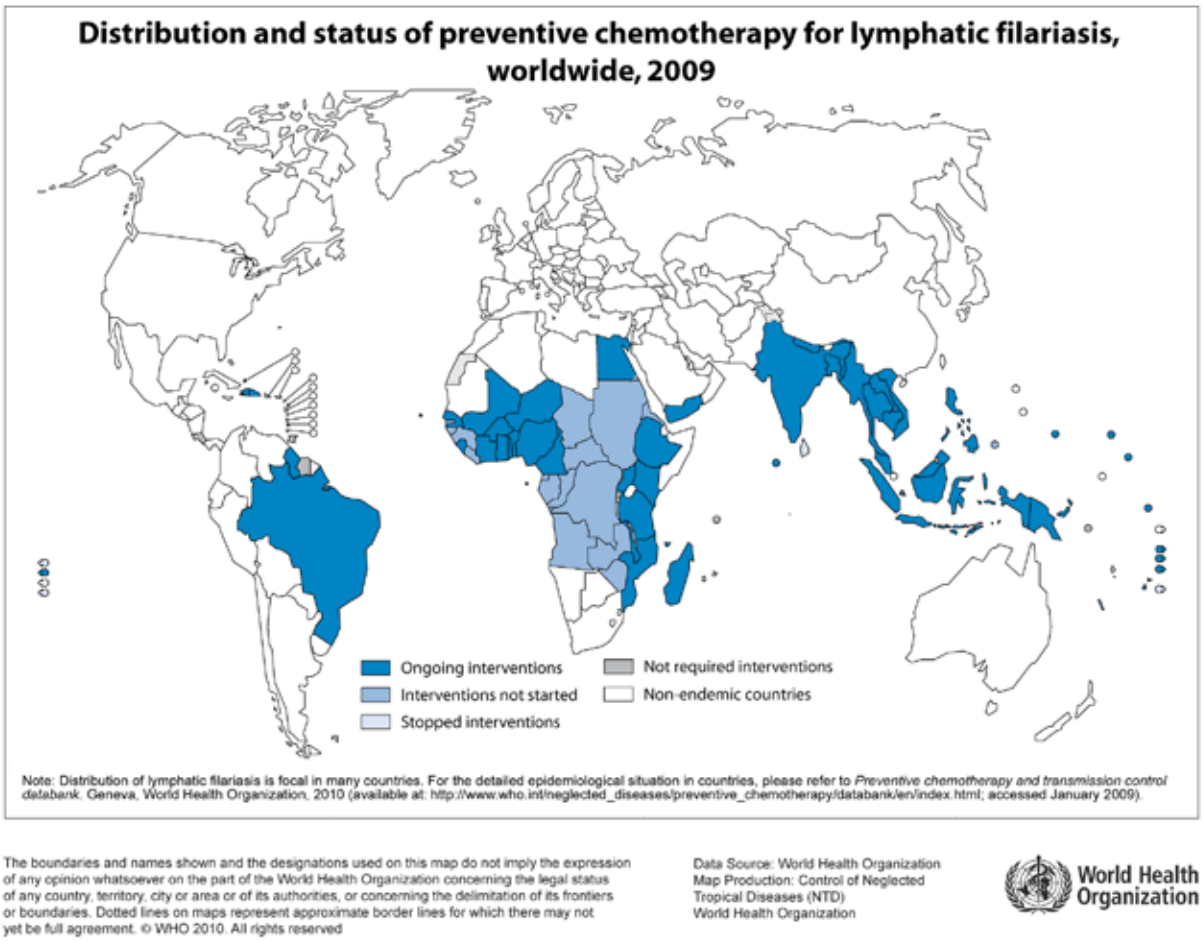

Figure 1. *The global distribution of lymphatic filariasis. Map sourced from the World Health Organization (WHO) based on the distribution of preventative chemotherapies to endemic regions in 2009 (http://gamapserver.who.int/mapLibrary/Files/Maps/Global_LF_2009.png). Reproduced by kind permission of the WHO.*

value of each approach differs depending on the stage of the parasite's lifecycle, the endemic region the patient has emigrated or visited, and if the patient is considered "endemic" or "non-endemic". A detailed flowchart of how to choose the appropriate diagnostic methods is outlined (Fig. 2).

Lifecycle

The lifecycle of the parasite (Fig. 3), which occurs in both the human and mosquito host, will influence the timing of sample collection and the interpretation of the results of the diagnostic tests. *W. bancrofti* and *B. timori* have only humans as the definitive host, while *B. malayi* can also occur in other mammals. Mf circulate in the blood of humans at night or day depending on the biting behaviour of the mosquito (Manguin *et al* 2010). Mf are ingested during a mosquito's blood meal. Host specificity for the mosquito is very low for all species of LF nematodes resulting in the parasites utilising many mosquito hosts globally. Within the mosquito the Mf undergo maturation into infective (L3) larvae at a rate that is temperature dependent and, unlike malaria, does not involve multiplication of the infective stage in the mosquito (Manguin *et al* 2010). L3 are not injected through the skin during a subsequent blood meal, but on to the skin where they have to find their way into the new host via the puncture wound made by the mosquito's proboscis, or through hair follicles; many larvae may be washed off the skin by sweat, or killed by dehydration (Manguin *et al* 2010). Thus transmission of LF is relatively inefficient, and it is estimated that between 2,500 and one million bites from an infective mosquito may be required before a patient infection is established (Southgate 1992). Efficiency is also strongly influenced by the species of vector and density of circulating Mf. *Aedes* species are able to effectively transmit LF when the density of circulating Mf is low, whereas *Anopheles* species require relatively high numbers of Mf (Bockarie *et al* 1998).

The larvae then travel to the lymphatics where they mature to the adult stage which is dioecious (males and females are separate). Development to the adult can take up to 12 months, during which time a person may be infected, but not have a "patent" infection (Bennuru and Nutman 2009b). Male and female adult worms mate and the female worm produces Mf, which enter the bloodstream where they are ingested by a female mosquito, completing the lifecycle.

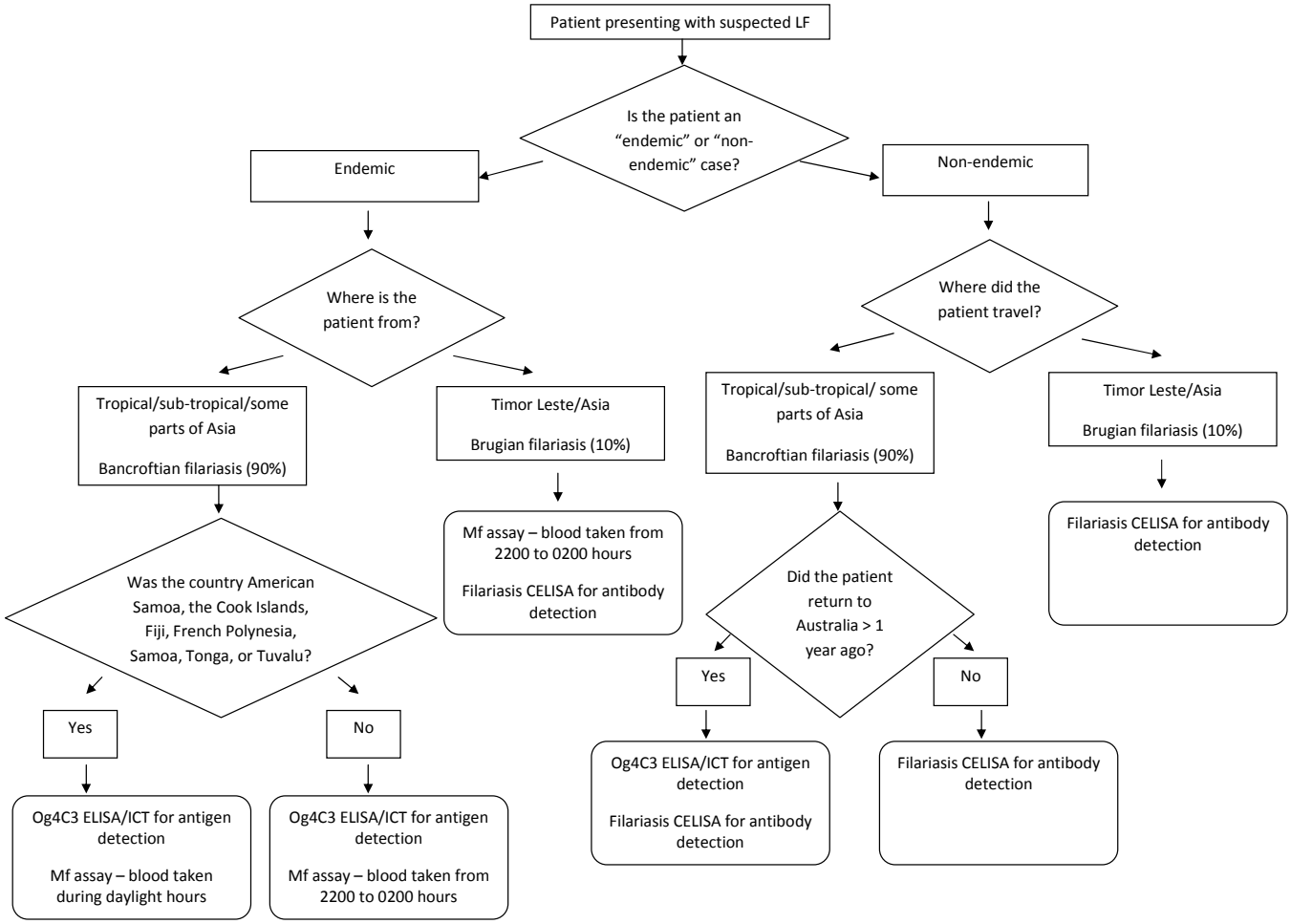

Figure 2. *Diagnostic flowchart for lymphatic filariasis.*

From the perspective of laboratory diagnosis, the important considerations are:

1. The morphology of Mf detected in blood can be used to identify the species of parasite (Orihel *et al* 1997); however, this is a specialised skill and difficult in the routine diagnostic laboratory.
2. Mf density in the peripheral blood shows periodicity. Thus blood samples should be collected at times to match the peak in Mf density. Periodicity means that the density of Mf per ml of peripheral blood varies with time of day. Different patterns of periodicity occur in different regions.
3. Not all patients infected with LF have Mf in the blood; infection may not be patent because the parasites are immature, worms of only one sex are present, females are effete and have stopped producing Mf, or the host immune response has cleared the Mf.
4. Antigen tests detect antigens associated with female worms capable of producing microfilaria; that is, infections with males only or dead females will be antigen negative.
5. There is a potential 12 month delay between infection and testing positive for infection by detecting Mf or circulating filarial antigen (CFA).
6. Serology can be positive a few months after exposure, but the nature of the antifilarial antibody varies with stage of LF and cross-reactivity can be a problem depending on the assay used.
7. A complete suite of diagnostic tests are available for *W. bancrofti*, but for *B . malayi* and *B. timori* detection of Mf and antibody serology are the only tests available.

Testing for microfilaraemia

Although detection of Mf is an old, well established method, it has been replaced by antigen tests for *W. bancrofti*, but not the other species (Weil and Ramzy 2007). However, for all species detection of Mf does indicate potential for transmission. If *W. bancrofti* infection is suspected, Mf detection is less sensitive than antigen tests as the initial choice for a diagnostic test. This is for three main reasons:

1. Only 50-70% of LF endemic cases are microfilaraemic because either the individual is infected with single sex worms, the female worms are immature and are incapable of reproduction, or the immune response has cleared the Mf from the circulation, or the females are effete and not reproducing (Njenga *et al* 2007).
2. Only approximately 0.2% of non-endemic LF cases have been reported as microfilaraemic (Wartman

1947). This is thought to be due to differences in immune sensitisation between endemic and non-endemic individuals (Ottesen 1992).

3. Unlike other diagnostic tests available, detection of Mf is dependent on the timing of the blood collection (Cox-Singh *et al* 2000). Timing of the peak levels of circulating Mf in the bloodstream vary among LF endemic regions and are thought to be dependent on the biting habits of the local mosquito vector (Manguin *et al* 2010). Where the mosquito is a nocturnal biter such as in Papua New Guinea, Mf appear in the blood during the night, usually peaking between 2200 hours and 0200 hours (Manguin *et al* 2010). In the diurnal form, found in areas where *Aedes sp.* is the vector such as Samoa, Mf appear in the blood during the day (Manguin *et al* 2010).

For *W. bancrofti* infection, if the patient tests positive for CFA, only then should an Mf test be performed because the presence of Mf will mean that there is at least the potential for transmission to another person. This is extremely unlikely in Australia, but if the patient is an expatriate of a country where LF has been eliminated and returns there before being treated there is a small chance that they could cause resurgence in their home country. This has been flagged as a concern in Pacific Island countries where the highly effective vector *Aedes polynesiensis* is present (Huppatz *et al* 2008).

Sample collection for Mf detection

Mf can be detected from either venous blood or finger prick blood. Venous blood is collected into an EDTA tube. Sample collection time will vary depending on the endemic region of origin and the periodicity of the parasite. Generally, if *Brugia*n filariasis is suspected, blood collection should occur at night between 2200 and 0200 hours. Blood collection times for bancroftian filariasis will differ (Fig. 2).

Diagnostic tools for detection of microfilariae

The commonly used diagnostic methods for detection of Mf include the thick smear, 3 line thick smear, the Knott's concentration technique, and membrane filtration. There are no commercially available assays for Mf detection. Each method has its own advantages and disadvantages. The thick smear is a typical malarial smear. The 3 line thick smear uses 3 lines of blood, each of approx 20 µL volume to give a total volume of 60 µL, placed in parallel longitudinally on a microscope slide. This allows for Mf density to be calculated. Both thick smears are stained using Giemsa stain and processed similarly to a malaria thick smear. The thick smear technique, particularly the 3 line thick smear, is easy to collect, stain, and results are 100% specific (Weil and Ramzy 2007). However, this technique is relatively insensitive because of the small volume of blood (60 µL) used and, in theory, can only detect a minimum density of 16.7 Mf/mL (Weil and Ramzy 2007). The filtration technique is effective in detecting small numbers of Mf, but forcing blood through a syringe and filter can create an aerosol hazard with the resultant risk of blood-borne infections.

The method of choice for a laboratory scientist in Australia using Mf is generally the Knott's concentration technique which uses 1 ml of venous blood. It has a sensitivity of 1 Mf/mL of blood, the formalin reduces the risk of infection, and once the blood-formalin dilution is made samples can be preserved for weeks without loss of detectable Mf (Melrose *et al* 2000). A detailed outline of how to perform the test is tabulated (Table 1). A modification of the Knott's test, using 2% Triton X-100, can save time and effort by making the sample easier to examine with no effect on Mf count, efficacy of Giemsa staining, or storage time of specimens (Melrose *et al* 2000).

Table 1. *Knott's Concentration Technique for microfilariae (Mf) detection (adapted from Orihel et al 1997)*

Materials and reagents	
1	Centrifuge tubes; 15 mL
2	Formalin, 2%
3	Slides and coverslips
4	Needles and syringes
5	Centrifuge and microscope
Method	
1	Collect 1 mL of venous blood into EDTA tube. Sample collection times will vary (Fig. 2).
2	Pipette into a centrifuge tube with at least 10 mL of formalin (2%)
3	Shake well to haemolyse the red blood cells
4	Centrifuge (500 g; 10 minutes)
5	Decant supernatant fluid (WBCs and Mf will be in pellet) and allow a small amount of fluid to flow back onto the sediment. Mix.
6	From the pellet, prepare a wetmount by placing a drop of the sediment on a slide under a coverslip. 1% Methylene blue can be added to the deposit, but isn't a necessity.
7	Examine under x20 and low light intensity
8	Additionally, Mf can be examined by thick and thin smears by fixing in methanol and staining with Giemsa.

Using Mf to identify the species causing infection

The morphology of the Mf can be examined to speciate the parasite. A diagnostic key based on the microscopic features of each of the species (*W. bancrofti*, *B. malayi* and *B. timori*) are tabulated (Tables 1 and 2). In addition, a diagnostic key can also be accessed from

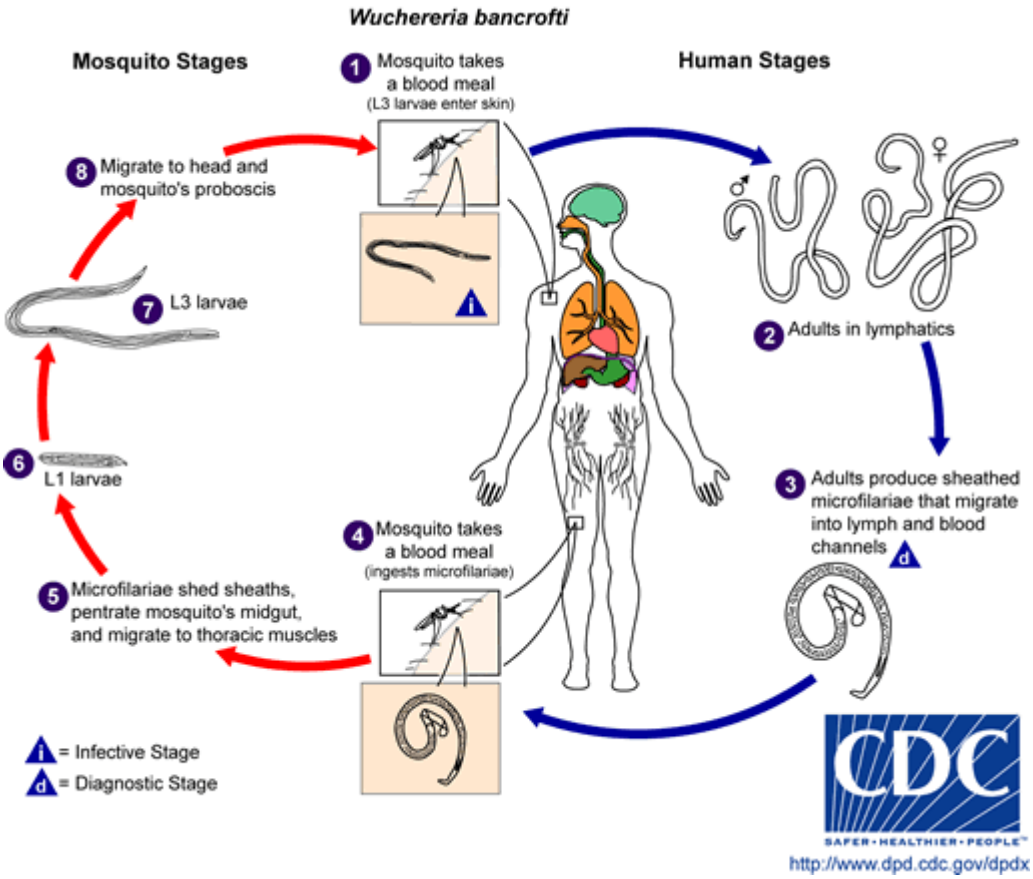

Figure 3. *The lifecycle of Wuchereria bancrofti. Lifecycle occurs in both a human host and the intermediate female mosquito host. Sourced from Centers for Disease Control and Prevention (CDC) (http://www.dpd.cdc.gov). Reproduced by kind permission of the CDC.*

the CDC website at *http://dpd.cdc.gov/DPDX/HTML/ImageLibrary/Filariasis_il.htm*

Interpretation of testing for Mf

A negative Mf assay does not exclude LF owing to its low sensitivity. Therefore, Mf testing should never be performed or interpreted to exclude LF independent of other diagnostic tests, particularly for *W. bancrofti* (Fig. 2).

Detecting the adult worm: Circulating Filarial Antigen (CFA) testing

The gold standard defined by the World Health Organization (WHO) for diagnosing LF infection is the detection of CFA (Melrose *et al* 2004). The CFA tests detect adult worm antigen in the bloodstream, indicating the presence of living adult worms in the host, and thus active infection (Weil *et al* 1999). Unfortunately, CFA assays only exist for the more common *W. bancrofti* species (Weil and Ramzy 2007). Thus other methods are required to detect *B. malayi* and *B. timori* infection.

Sample collection for CFA detection

Samples for CFA detection can either be venous blood collected into an EDTA tube or serum. Samples can be collected at any time, unlike the nocturnal or diurnal requirement for Mf testing.

Table 2. *Key microscopic features of microfilariae (Mf) (adapted from Orihel et al 1997)*

Species	*Wuchereria bancrofti*	*Brugia malayi*	*Brugia timori*
Length (μm) Smears 2% formalin	 244-296 (260) 275-317 (298)	 177-230 (220) 240-298 (270)	 265-323 (287) 332-383 (358)
Width (μm)	7.5-10.0	5.0-6.0	4.4-6.8
Tail	Tapered; Anucleate	Tapered; Subterminal and terminal nuclei widely separated	Tapered; Subterminal and terminal nuclei widely separated
Key features	Short head space; Dispersed nuclei; Body in smoothed curves	Long head space; Terminal and subterminal nuclei	Long head space; Terminal and subterminal nuclei
Key features if stained with Giemsa	Sheath unstained	Sheath stains pink	Sheath unstained

Diagnostic tools for CFA detection

CFA detection tools commercially available for *W. bancrofti* are the Binax NOW® Filariasis Immunochromatographic Test (ICT) (available from www.binaxnow.com; Australian distributor Alere) and the Trop-Ag *W. bancrofti* ELISA

(available from Trop Bio, Pty. Ltd., Australia www.tropbio.com.au) (Turner *et al* 1993; Weil *et al* 1997). The former is a rapid card test format based on specific monoclonal (AD-12) and polyclonal antibodies for a 200kDa antigen (Weil *et al* 1997; Schuetz *et al* 2000); the latter is an ELISA based on a monoclonal IgM antibody (Og4C3) raised against a bovine *Onchocerca gibsoni* antigen with high specificity for a *W. bancrofti* antigen (Weil *et al* 1997; Melrose 2004). A recent study demonstrated the same sensitivity (97%) for both assays, but specificity was not determined (Rocha *et al* 2009). Currently, the Trop-Ag *W. bancrofti* ELISA is only available as a 96-well plate format, designed to test either 80 samples or 40 duplicate samples (Tropbio Pty Ltd, Townsville). Therefore, cost per test increases markedly if only small numbers of tests are performed. Given this, the most appropriate and convenient laboratory CFA diagnostic tool for *W. bancrofti* in Australian diagnostic settings is the ICT (Rocha *et al* 2009). The ICT is easy to perform, practical and quick.

The ICT is based on capillary migration of approximately 100 µL of whole blood/serum. The blood migrates down the card, and any *W. bancrofti* antigen present in the blood sample binds to the polyclonal or monoclonal antibodies impregnated in the filter of the card, thereby producing a visible reaction (Fig. 4). The assay is quick and easy to perform, but the result must be read at exactly 10 minutes to avoid false positives (Rajgor *et al* 2002).

Reported sensitivities for the ICT range from 65% to 98% (Weil *et al* 1997; Ramzy *et al* 1999; Rocha *et al* 2009), with variations dependent on whether the individual is Mf positive and the level of antigenaemia. The original specifications for the assay were reported as: sensitivity of 91% (95%CI 86 – 95), specificity of 99% (95%CI 97 – 100), positive predictive value of 99% (95%CI 97 – 100), and negative predictive value of 91% (95%CI 86 – 94).

Figure 4. *The Binax NOW ® Filariasis Immunochromatographic Test (ICT). A positive test is represented by a line at both the test (T) and control (C) in the viewing window.*

Interpretation of CFA testing

Whether the laboratory has utilised the Trop-Ag *W. bancrofti* ELISA or the ICT, the interpretation of the result is critical. A positive result will indicate that the person is infected with an adult worm. On the contrary, a negative result does not necessarily exclude infection, since the length of the lifecycle results in a delay of up to 12 months before an infected person tests positive (Sasa 1976). In this case, if LF is strongly suspected and the patient originates or returns from a *W. bancrofti* endemic area, then testing for antigen should be repeated 12 months later.

Detecting exposure: antibody testing

Anti-filarial antibody assays were developed as early as 1980, and included a wide range of crude filarial parasite antigens and whole parasite lysates (Melrose 2004). These assays had low specificity because of cross-reactivity with other helminths such as hookworm and *Strongyloides* sp. (Melrose 2004). This was a problem in LF endemic areas since poly-parasitism is common (Utzinger and de Savigny 2006). Advances in recombinant antigen technology have increased both sensitivity and specificity, specifically through the use of the recombinant antigen *B. malayi* 14 (Bm14) (Lammie *et al* 2004; Weil *et al* 2010; Joseph *et al* 2011a; Joseph *et al* 2011b). A commercial assay, the Filariasis Ab (Bm14) CELISA, based on Bm14 is available (Cellabs Pty Ltd, Manly, Australia).

Despite the availability of more specific antibody diagnostic methods using recombinant antigen, LF diagnosis in Australia generally relies on less specific techniques that uses somatic antigens from whole *Dirofilaria immitis* (dog heartworm) (Jeremiah *et al* 2011). It is desirable that LF antibody testing uses the more accurate techniques based on recombinant antigens.

Sample collection for antibody detection

Samples for antibody detection can either be plasma collected into an EDTA tube or serum. Samples can be collected at any time, unlike the nocturnal or diurnal requirement for Mf testing.

Diagnostic tool for antibody detection

The Filariasis Ab (Bm14) CELISA is commercially available from Cellabs Pty Ltd, Sydney (www.cellabs.com.au). The assay has been demonstrated to be highly sensitive and specific using serum/plasma (Lammie *et al* 2004; Joseph and Melrose 2010; Weil *et al* 2010) and performed well in public health surveys (Joseph 2010; Joseph *et al* 2011a; Joseph *et al* 2011b). The instructions for the ELISA are easy to follow, are based on the H_2O_2 peroxidase detection system, and plates are read at a dual wavelength of 450/650 nm. Additionally, the

recombinant antigen utilised (Bm14) cross-reacts with all of the filarial species, making the assay useful for detecting exposure to both *W. bancrofti* and *Brugia* spp (Lammie *et al* 2004). This is especially useful because of the lack of an antigen test for the *Brugia* spp.

Interpretation of antibody testing

Interpretation of the antibody test depends upon the patient history. As discussed, non-endemic patients who are infected could potentially test negative for Mf and CFA because of the pre-patent phase in the parasite lifecycle. Consequently, for a non-endemic patient, detection of specific antibodies in the absence of the other parameters may be sufficient to confirm a diagnosis. Interpretation of the antibody results for endemic patients is more complicated. A positive result for an endemic patient from a *W. bancrofti* endemic area would be expected regardless of infection status because of the ongoing exposure to infective mosquitoes (Njenga *et al* 2007). However, a positive result for an endemic patient from a *B. malayi* endemic area should be treated as though potentially infected since confirmatory antigen testing is not available.

Interpretation of negative samples

If the patient tests negative for microfilaraemia, antigenaemia, and antibodies this does not necessarily exclude a diagnosis for LF since those patients with end-stage chronic pathology (chronic lymphoedema, elephantiasis and hydrocele) are often no longer infected and also test negative for antibodies (Weil *et al* 1987; Ramzy *et al* 1991). It is thought that the ongoing manifestations of disease during this uninfected chronic stage are due to damage of the lymphatics and immunopathology (Morchon *et al* 2008; Bennuru and Nutman 2009a). Diagnosis of these patients relies on clinical considerations and not on laboratory methods. Therefore, it is imperative that the laboratory scientist communicates negative findings with this information so that the referring doctor can make an informed decision based on all clinical findings.

Costs of laboratory tests and Medicare billing status

Medicare billing for filariasis serology can be charged under general serological testing for infectious organisms. The current rebate from Medicare for such testing is approximately $15 per sample.

Microfilaraemia testing

The costs involved with performing the test include laboratory consumables and labour.

Circulating filarial antigen testing

As previously mentioned, the Trop-Ag *W. bancrofti* ELISA is only available as a 96-well plate format, designed to test either 80 samples or 40 duplicate samples. However, the manufacturer is currently considering altering the ELISA to a strip-well format which should reduce costs. Currently, cost per singular sample is approximately $17.70 when duplicate controls are included. Assaying more samples at one time would reduce the costs slightly, as the controls are per 96-well plate. The ICT costs approximately $6.80 per test.

Antibody testing

The ELISA is a 96-well plate format, but can be tested in individual strips of 8 wells. Cost per sample is approximately $14.58 including controls. Assaying more samples at one time would reduce the costs slightly.

Summary

This review describes currently available diagnostic methods for the detection of LF. Sample collection, global distribution of the parasites responsible for LF, differences in diagnosing endemic versus non-endemic patients, and test interpretation are covered. Misdiagnosis of LF can be common due to inappropriate sample collection and diagnostic methodology used. By referring to this review and the diagnostic flowchart, the laboratory will have an accurate and up-to-date document for guidance if presented with a request for LF testing.

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