LYMPHATIC FILARIASIS ELIMINATION: RESIDUAL ENDEMNICITY, SPATIAL CLUSTERING AND FUTURE SURVEILLANCE USING THE NEW FILARIASIS CELISA DIAGNOSTIC ASSAY

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in June, 2010

in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the School of Public Health, Tropical Medicine and Rehabilitation Sciences, James Cook University of North Queensland, Australia

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I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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PREFACE

The research was made possible by ongoing financial support from GlaxoSmithKline (GSK). By using the statistical software, SaTScan, I am obligated to note: "SaTScan™ is a trademark of Martin Kulldorff. The SaTScan™ software was developed under the joint auspices of (i) Martin Kulldorff, (ii) the National Cancer Institute, and (iii) Farzad Mostashari of the New York City Department of Health and Mental Hygiene".

The research undertaken as part of the thesis was a collaborative effort with the Pacific Programme for the Elimination of Lymphatic Filariasis (PacELF), based in Fiji, and the Centers for Disease Control and Prevention (CDC), based in USA. The bulk of the research from Samoa was a collaborative effort with the World Health Organization (Apia, Samoa) and the Ministry of Health in Samoa.

In general, editorial assistance was provided by my supervisors including advice on data interpretation. Advice on statistical analysis is described in detail below.

Base Shape Files for ArcGIS Version 9.0
Phil Bright (Secretariat to the Pacific Community (SPC), New Caledonia).

Finalisation of Maps (only Samoa)
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Julian Lawn (JCU, Australia)

Spatial Statistics
Shannon McClintock (CDC, USA)
James Moloney (JCU, Australia)

Categorical and Numerical Statistical Analysis
Petra Buttner (JCU, Australia)
Alan Clough (JCU, Australia)

ACKNOWLEDGEMENTS

I would like to thank all of the people in the Pacific Islands who participated in this study. If it were not for these people my work would not have been possible.

PhDs are not successful without the guidance and support provided by the student's academic advisors. I was fortunate enough to be advised by Professor Richard Speare, Dr Patrick Lammie and Associate Professor Wayne Melrose. I would like to thank Rick for his guidance with the thesis writing. The man is a mountain of knowledge! I would like to thank Pat for his help with thesis writing, publications and as a sounding post for some of the crazy ideas I've come up with over the past few years. Pat helped me a lot with the spatial studies and I enjoyed "picking his brain" during my six week stay at the CDC, USA.

It is difficult to explain in words the impact my principal supervisor, Associate Professor Wayne Melrose, has had on me both professionally and personally. I am forever indebted to him for his invaluable supervision and advice over these years, which have been paramount in moulding the research scientist I am today. His unconventional approach to science and love of controversial findings has fuelled my interest and love of this topsy-turvy world of research. Wayne is an exceptional supervisor. He always gave me "just enough rope" not to hang myself; he always gave me the space to learn, grow, and pushed me out of that comfort zone, but not too much where I would be left flailing! I will never forget organising my first field trip in the Pacific, where I would be team leader. Wayne was away and I was left to organise all supplies. I was nervous and "wet behind the ears" and didn't know what to expect. I certainly was not used to such isolated conditions. I recall emailing Wayne a series of questions and Wayne (who by this stage was well aware of my over-preparation and anal retentiveness) emailed me back. His email did not consist of answers to my questions, but rather a short and sweet email of: "STOP WORRYING! I am confident in your abilities and have no doubt you will sort out these problems when you're on

the ground…… oh by the way, do not forget the emergency beacon on my desk – you may need it….." And believe me, there were times when I felt like flicking that switch! Running my own field teams in various countries across the Pacific has provided me with priceless life lessons and memories and I only have Wayne to thank for providing me with this opportunity.

I would like to thank the lovely administrative staff; Ari and Michelle. Especially for never deleting my emails that started with "I have a small favour to ask……" or ignoring me when I came in to work and began a sentence with "could you hold Chloe while I……." (Although I am suspicious they loved the second statement so they could get some cuddles! And Chloe, my baby, loved it too!). Furthermore, I would like to thank the Trisha for helping with those fiddly end stages of printing.

Of course I am saving "the best" until last. The constant support of my parents has been paramount and integral to my successes not only in the postgraduate stage of my career, but also my undergraduate study at JCU. The emotional and professional guidance provided by my parents has helped shape the individual I am today. During those times of hardship, which I am sure each PhD student has endured, the love and support of both my parents always saw me through. Furthermore, I will always be appreciative of the endless babysitting provided by my mum during the hours of thesis writing! Secondly, the hours of editing by my dad have greatly improved the readability of the thesis and to him I will always be grateful. Now, dad, as a grandad it's time to help Chloe with her years of schooling!

To my sister and brother-in-law, Rachel and Mat, you may never quite understand the point of my research, but you were always willing to listen to my rants and complaints when things went wrong and revelled in my jubilation when things succeeded. Furthermore, Rachel helped me with my "thesis-free" days to keep me sane and let our beautiful children play happily together.

Most importantly, I would like to thank my husband, Deep, and my beautiful daughter, Chloe. Deep: how you managed to convince me that a PhD is a good idea I will never know! Now that I am at the end, and have learnt a great deal, I realise that I would never have made it this far without you. You never doubted my abilities and always provided cuddles to put a smile on my face. Chloe: you're an angel of a baby. I would never have been able to get so much thesis writing done without your calm nature. You were always easily comforted and quite happy to occupy yourself with your toys while I madly typed away on the computer. You loved being in the sling on my days in at JCU printing off pages and pages of drafts (and loved even more ripping those drafts to shreds when I was finished with them!). You loved listening to me while I read aloud excerpts of my chapters and giggled when I used my "funny" voice. Let's hope your first words aren't "lymphatic filariasis"…… I love you both dearly. Happy reading!

PRESENTATIONS

Joseph, H., and Melrose, W. Laboratory Diagnosis of Lymphatic Filariasis. Australian Institute of Medical Scientists (AIMS), Cairns, Australia, June 2007

Joseph, H., Lymphatic Filariasis in Samoa; project proposal. Ministry of Health, Samoa, May 2008

Joseph, H., Lammie, P., McClintock, S., Maiava, F., and Melrose, W. Spatial Analysis of Lymphatic Filariasis in Samoa. American Society of Tropical Medicine and Hygiene (ASTMH) conference, New Orleans, USA, Dec 2008

Future Presentations:

Joseph, H., Lammie, P., McClintock, S., Maiava, F., and Melrose, W. First Evidence of Spatial Clustering of Lymphatic Filariasis in an ***Aedes polynesiensis*** Endemic Area. American Society of Tropical Medicine and Hygiene (ASTMH) conference, Atlanta, USA, Nov 2010

PUBLICATIONS

Joseph, H., and Melrose, W. D. (2010) Applicability of the filter paper technique for detection of antifilarial IgG_4 antibodies using the Bm14 Filariasis CELISA. ***Journal of Parasitology Research*** doi:10.1155/2010/594687: 6 pages

Weil, G.J., Curtis, K.C., Fischer, P.U., Won, K.Y., Lammie, P.J., **Joseph, H.M.,** Melrose, W.D., and Brattig, N.W., (2010). A multi-centre evaluation of a new antibody test kit for lymphatic filariasis employing recombinant ***Brugia malayi*** antigen BM-14. ***Acta Tropica*** [Epub ahead of print]

Dos Santos, M., Armaral, S., Harmen, S., **Joseph, H.,** Fernandes, J. and Counahan, M. (2010). The prevalence of common skin infections in Timor-Leste: A cross sectional survey. ***BMC Infectious Diseases*** **10**: 61

Future Publications

Joseph, H., Maiava, F., Lammie, P. and Melrose, W. Evaluation of continuing transmission and clustering of residual infection of lymphatic filariasis in Samoa. Part I: epidemiological assessment ***In Submission Acta Tropica***

Joseph, H., Moloney, J., Maiava, F., McClintock, S., Lammie, P. and Melrose, W. Evaluation of continuing transmission and clustering of residual infection of lymphatic filariasis in Samoa. Part II: spatial clustering ***In Submission Acta Tropica***

Joseph, H., Clough, A., Maiava, F and Melrose, W. Exploratory Study Investigating Factors Influencing Mass Drug Administration (MDA) Compliance for Lymphatic Filariasis in Samoa, ***Manuscript in preparation***

Joseph, H., Moloney, J., Maiava, F., Taleo, F., 'Ake, M., Capuano, C. and Melrose, W. Application of the Filariasis CELISA anti-filarial IgG_4 antibody assay in LF surveillance in the South Pacific. ***Manuscript in preparation***

ABSTRACT

Lymphatic Filariasis (LF) is a mosquito-transmitted parasitic disease caused by the filarial nematodes ***Wuchereria bancrofti***, ***Brugia malayi*** and ***Brugia timori***. In 1997, the 50th World Health Assembly approved a resolution calling for the elimination of LF as a public health problem (WHA50.29). This was deemed achievable with a regime of annual Mass Drug Administrations (MDAs) and, where appropriate, vector control for a minimum of four to six years. The Pacific counterpart was named the Pacific Programme to Eliminate Lymphatic Filariasis (PacELF). In the Pacific, countries which have reached the threshold of < 0.1% circulating filarial antigen (CFA) prevalence in children entered surveillance mode until 2012, whereas countries with persistent transmission planned further MDAs. Successful elimination of LF requires:

1) Accurate identification of residual foci of transmission (in countries with persistent transmission);
2) Comprehensive surveillance strategies to detect and combat potential resurgence (in countries entering surveillance mode); and,
3) Culturally appropriate education campaigns to encourage MDA compliance, as systematic non-compliers become reservoirs of infection.

It is crucial to apply sensitive diagnostic tools which are capable of identifying these areas of residual endemnicity or resurgence early. This phase of low prevalence poses particular challenges: “hot spots” may be scattered and ill-

defined and the diagnostic tools measuring microfilaraemia (Mf) and CFA that were successful in the earlier phase of the programme may no longer be adequate because of issues with sensitivity, the requirement for larger sampling sizes, and lag phases before Mf or CFA are detectable in newly infected persons. The addition of antibody serology as a complementary diagnostic tool would provide an earlier warning system, since children born after the interruption of transmission would be antibody negative.

In order to incorporate serology into the LF programme, use of a standardised commercial assay must be used, such as the Filariasis Cellabs Enzyme-Linked Immunosorbent Assay (CELISA). Although the Filariasis CELISA has been manufactured since 2006, it is yet to be investigated for its potential use in large scale sampling. It was the aim of this research to determine:

1) The efficacy of the Filariasis CELISA antibody assay;
2) Its usefulness as a potential diagnostic tool for the inclusion into the LF programme; and,
3) Its role in future surveillance work.

This was achieved by validating the Filariasis CELISA for field applicability, assessing its efficacy for identifying areas of residual endemnicity, and investigating the spatial relationships between exposed and infected individuals. In addition, during the progression of the thesis, data became available concerning MDA compliance in Samoa. MDA compliance is also crucial for successful elimination of LF since systematic non-compliers remain as potential reservoirs of infection.

The Filariasis CELISA was easily applicable for field work using whole blood dried onto filter paper. Filter paper sampling had a sensitivity of 92% and a specificity of 77%, when compared to plasma samples. Five thousand four hundred and ninety-eight filter paper samples were assayed from four LF endemic South Pacific countries (Tuvalu, Tonga, Vanuatu, and Samoa). Antibody prevalence rates correlated with cessation of LF transmission in Tonga and Vanuatu, both of which have entered surveillance mode, and ongoing transmission in Samoa and Tuvalu. Most importantly, use of CFA prevalence in children alone, the current World Health Organization (WHO) recommendation, missed vital residual areas of endemic foci in Samoa, as observed by high antibody prevalence in children and Mf positive individuals. This observation required further investigation with an in-depth epidemiological study.

In Samoa, five villages were chosen for prevalence surveys, including Siufaga, which was originally believed to be LF-free. Results showed that the reservoir of infection was the older males and that there was a correlation between transmission (Mf/CFA positivity) and exposure in children. Crucially, ongoing transmission was occurring in Siufaga, as demonstrated by an overall CFA prevalence exceeding 1% and high antibody prevalence in children. CFA testing of children alone would not have identified Siufaga as an area of residual endemnicity.

Accurate identification of residual foci of transmission is challenging in areas where ***Aedes polynesiensis*** is endemic, such as Samoa, since no

geographical clustering of infection has been demonstrated. Results from the aforementioned epidemiological study were spatially linked to household of residence (community based analyses) and/or primary school (school based analyses) of attendance. “Community based” analyses revealed significant spatial clusters of households with infected individuals and a relationship to antibody positive children when they were included in the spatial analysis. Similar results were observed for “school based” analyses. These promising findings are the first evidence of spatial clustering of LF in a day-biting ***Ae. polynesiensis*** endemic area. In addition, these results are the first evidence of dual clustering of Mf/CFA individuals with exposed children.

In Samoa, MDA non-compliance of infected individuals may contribute to persistent transmission. Exploring why these individuals are non-compliant is of paramount importance to the LF programme. Individuals testing positive for LF and children aged 7 – 10 years were asked to participate in a questionnaire designed to ascertain: 1) level of LF knowledge, (2) compliance, and (3) a number of risk factors. For the infected individuals, there was a significant association between MDA compliance and knowledge of LF and, for the children, this association also extended to use of mosquito protection. This exploratory study highlights the need for restructuring current educational campaigns, and their deliverance, to appropriately target children and the systematically non-compliant infected individuals. In addition, the study highlights the necessity to instigate qualitative studies to explore cultural and religious beliefs; a strong driver of compliance.

The overall findings fill critical gaps in knowledge for the elimination of LF namely:

1) Incorporation of antibody serology should be a priority because:
    a. Certain areas of residual transmission will not be detected using Mf or CFA diagnostic testing alone; and,
    b. Surveillance requires a diagnostic test capable of detecting resurgence early so that action can be timely.
2) In Samoa:
    a. Identification of spatial clustering has a significant impact on the LF programme in terms of targeted treatment, re-introduction of vector control campaigns and aiding health personnel to locate potential Mf positive cases;
    b. Previously declared “LF-free” villages may have residual transmission; and,
    c. New health education campaigns are a necessity for targeting non-compliant individuals.

The addition of antibody serology into the repertoire of LF diagnostic tools holds huge promise for identifying areas of residual endemnicity and in future surveillance and control of LF.

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LIST OF ABBREVIATIONS

Ab	antibody
ADCC	antibody dependent cellular cytotoxicity
ADL	adenolymphangitis
ADLA	acute dermatolymphangioadenitis
AFL	acute filarial lymphangitis
APCs	antigen presenting cells
Bm	***Brugia malayi***
Bm14	***Brugia malayi*** 14
BmR1	***Brugia malayi*** recombinant antigen 1
X^2	Chi-squared
C	control
CDC	Centers for Disease Control and Prevention
CELISA	Cellabs enzyme linked immunosorbent assay
CFA	circulating filarial antigen
CI	confidence intervals
COMBI	Communication for Behavioural Impact
CTS	child transmission survey
DEC	diethylcarbamazine citrate
DNA	deoxyribonucleic acid
^oC	degrees Celsius
df	degrees of freedom
DOT	directly observed therapy
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
g	gravitational force
GIS	geographic information system
GMP	good manufacturing practice
GPELF	Global Programme for the Elimination of Lymphatic Filariasis
GPS	global positioning system
GSK	GlaxoSmithKline
HH	household
HIV	human immunodeficiency virus

H_2O_2	hydrogen peroxide
ICT	immunochromatographic test
IEC	information, education and communication
IFNg	interferon gamma
Ig	immunoglobulin
IL	interleukin
iL3	infective larvae stage three
IU	implementation unit
JCU	James Cook University
KAP	Knowledge, Attitudes and Practices
kDa	kilodalton
kg	kilogram
km	kilometre
LF	lymphatic filariasis
LPS	lipopolysaccharide
LQAS	lot quality assurance sampling
m	metres
MDA	mass drug administration
µg	microgram
mg	milligram
µL	microlitre
mL	millilitre
Mf	microfilariae
n	number of participants
N/A	not applicable
ND	not done
NSW	New South Wales
NK	natural killer
nm	nanometres
NMEA	National Marine Electronics Association
NO	nitric oxide
NPV	negative predictive value
NTD	neglected tropical disease
OCP	onchocerciasis control programme

OD	optical density
Og4C3	***Onchocerca gibsoni*** 4C3
OPD	***o***-phenylenediamine
OR	odds ratio
PacCARE	PacELF Coordination and Review Group
PacELF	Pacific Programme for the Elimination of Lymphatic Filariasis
PAR	participatory action research
PBS	phosphate buffered saline
PC	phosphorylcholine
PCR	polymerase chain reaction
PICT	Pacific Islands Countries and Territories
PNG	Papua New Guinea
PPV	positive predictive value
QC	quality control
Rad	radius
RMS	root mean square
RNA	ribonucleic acid
RR	relative risk
RTPCR	reverse transcriptase polymerase chain reaction
SD	standard deviation
SPC	Secretariat to the Pacific Community
X	times
T	test
Th	T helper cell
TMB	3,3',5,5'-tetramethylbenzidine
TNFα	tumour necrosis factor alpha
TV	television
VIC	Victoria
vs	versus
Wb	***Wuchereria bancrofti***
WHA	World Health Assembly
WHO	World Health Organization

CHAPTER 1

GENERAL INTRODUCTION

Lymphatic filariasis (LF) is a mosquito-transmitted parasitic disease caused by the filarial nematodes ***Wuchereria bancrofti***, ***Brugia malayi,*** and ***Brugia timori*** (Ottesen, 2006). In 1997, during the 50th World Health Assembly (WHA), a resolution was approved calling for the global elimination of LF as a public health problem (WHA50.29) (WHO, 2005). The resolution acknowledged the morbidity and socioeconomic costs of LF, including the general lack of awareness of the disease and the potential for its eradication (CDC, 1993). In 1999, under the direction of the World Health Organization (WHO), the Global Programme for the Elimination of LF (GPELF) was developed and was based on a comprehensive strategy to rid countries of LF as a public health problem by the year 2020 (WHO, 2005). This included the interruption of LF transmission and morbidity control for those already afflicted (WHO, 2000). The Pacific counterpart of GPELF was named the Pacific Programme for the Elimination of LF (PacELF) (Ichimori and Crump, 2005; PacELF, 2006).

Prevalence of Bancroftian LF, caused by ***W. bancrofti***, throughout the Pacific was historically high (PacELF, 2006). Sixteen of the 22 countries falling under the jurisdiction of PacELF were classified as endemic following baseline prevalence surveys. They were: American Samoa, the Cook Islands, the Federated States of Micronesia, Fiji, French Polynesia, Kiribati,

the Marshall Islands, New Caledonia, Palau, Papua New Guinea (PNG), Samoa, Solomon Islands, Tonga, Tuvalu, Wallis and Futuna, and Vanuatu (WHO, 2007a). Originally, PacELF set the target of LF elimination as a public health problem by 2010, demonstrated by < 1% circulating filarial antigen (CFA) prevalence of the population or < 0.1% CFA prevalence in children (PacELF, 2006; Ichimori ***et al.***, 2007a). The strategic plan focussed on the scheduling of a minimum of five rounds of mass drug administration (MDA), with vector control where appropriate, depending on baseline prevalence in each country and the results of post-MDA surveys (PacELF, 2006; Ichimori ***et al.***, 2007a). Since then, post-campaign prevalence surveys have revealed persistent ongoing transmission in certain countries suggesting the need to intensify efforts (Chanteau and Roux, 2008; Huppatz ***et al.***, 2009). Subsequently, at the 2007 PacELF meeting, a five year plan was drafted (WHO, 2007a). Countries on the brink of elimination entered monitoring and surveillance mode until 2012, whereas other countries with ≥ 1% CFA prevalence planned further control efforts (WHO, 2007a). The target date for the elimination of LF from PNG was extended until 2020 (WHO, 2007a). This was due to the high infection rates observed in this country and the limited progress for implementing the national programme for LF elimination (WHO, 2007a). This was predominantly due to economic and political problems (WHO, 2007a).

The challenges now facing the LF programme include:

1) Identifying areas of residual endemnicity for further control efforts; and,
2) Implementing efficient strategies for ongoing surveillance;

To assess the effectiveness of elimination efforts globally, as well as implement successful surveillance strategies in previously LF endemic countries, it is crucial to apply sensitive diagnostic tools which are capable of identifying these areas of residual endemnicity or resurgence early. WHO currently approves the use of diagnostic methods to detect microfilariae (Mf) and CFA. These diagnostic methods have proven to be extremely useful during the elimination programme stages, where parasite prevalence is relatively high, but there is concern that they might not be sensitive enough to detect residual endemnicity or resurgence in the post-programme phase (Weil and Ramzy, 2007; Huppatz ***et al.***, 2008).

This phase of low prevalence poses particular challenges: “hot spots” may be scattered and ill-defined and the diagnostic tools measuring Mf and CFA that were successful in the earlier phase of the programme may no longer be adequate because of issues with sensitivity, the requirement for larger sampling sizes, and lag phases before Mf or CFA are detectable in newly infected persons (Burkot ***et al.***, 2002; Ramzy, 2002; Durrheim ***et al.***, 2003; Lammie ***et al.***, 2004; Melrose ***et al.***, 2004; Rawlins ***et al.***, 2004; Washington ***et al.***, 2004; Grady ***et al.***, 2007; Weil and Ramzy, 2007; Ramaiah ***et al.***, 2009). The addition of antibody serology as a complementary diagnostic method when prevalence is low may provide an earlier warning system

(Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009).

Antibody responses to LF exposure have been suggested as an ideal immunological measurement for identifying areas of residual endemnicity or in future surveillance campaigns (Lammie ***et al.***, 2004; Washington ***et al.***, 2004; Ramzy ***et al.***, 2006; Grady ***et al.***, 2007; Njenga ***et al.***, 2007a). Previous studies demonstrated that antibody production in response to LF exposure occurs during the first few years of life (Gao ***et al.***, 1994) and, therefore, if cessation of LF transmission has occurred then children should be antibody negative (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). In order to incorporate serology into the LF programme a standardised commercial assay must be used, such as the Filariasis Cellabs Enzyme Linked Immunosorbent Assay (CELISA) (Weil ***et al.***, 2010).

The Filariasis CELISA measures anti-filarial immunoglobulin (Ig) G_4 against the recombinant antigen ***B. malayi*** 14 (Bm14) and has been commercially available since 2006 (Cellabs Pty Ltd., Manly, Australia). The research-based Bm14 assay prototype has had promising results detecting Ig in response to both ***Brugia*** sp. and ***W. bancrofti***, with cross-reactivity observed for both ***Loa loa*** and ***Onchocerca volvulus*** (Weil ***et al.***, 1999; Lammie ***et al.***, 2004; Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). However, the efficacy of the commercial assay in large-scale field

sampling is yet to be ascertained. However, in laboratory-based experiments, the Filariasis CELISA has higher sensitivity and specificity than the research-based Bm14 assay prototype as well as little interlaboratory variation (Weil ***et al.***, 2010). This highlights its potential use for future inclusion into the available repertoire of LF diagnostic tools.

Sensitive diagnostic tools are not the only requirement in this challenging phase. Innovative sampling methods are also needed to maximise the likelihood of identifying problem areas whilst taking into consideration resource and economic restraints (Ramaiah ***et al.***, 2009). These sampling methods could encompass:

1) Seroprevalence mapping (Booth and Dunne, 2004);
2) Fine scale spatial mapping with spatial statistical software (Brooker ***et al.***, 2006; Raso ***et al.***, 2006a; Raso ***et al.***, 2006b; Matthys ***et al.***, 2007; Raso ***et al.***, 2007; Pullan ***et al.***, 2008; Brooker and Clements, 2009; Clements ***et al.***, 2009; Eisen and Lozano-Fuentes, 2009); and,
3) Sampling children (first year level school entrants) born after cessation of LF transmission (WHO, 2005).

The challenge for using fine scale spatial mapping in the Pacific is the presence of the day-biting ***Aedes polynesiensis*** vector, which is endemic in certain Pacific countries. Unlike the other mosquito vectors of LF, no spatial clustering has been detected in areas where ***Ae. polynesiensis*** is the predominant vector (Cuenco ***et al.***, 2009; Mladonicky ***et al.***, 2009). This was thought to be due to population movement during daylight hours (Mladonicky

et al., 2009). This makes defining geographical areas of ongoing transmission a potential challenge. Evidence of clustering would be useful for guiding surveillance efforts.

Finally, identifying areas of residual endemnicity, through the use of sensitive diagnostic methods and innovative sampling, requires further investigation to ascertain why these areas exist. Successful elimination has been linked to community involvement and MDA compliance (PacELF, 2005; Kyelem ***et al.***, 2008; Alemayehu ***et al.***, 2010), where untreated cases remain as disease reservoirs (Mathieu ***et al.***, 2006; Boyd ***et al.***, 2010). Therefore, there is the need for further information and understanding about issues surrounding MDA compliance.

The overall aim of this research was to assess the feasibility of using antibody serology, as measured with the Filariasis CELISA, to identify endemic foci and to complement future surveillance strategies. To achieve the overall aim, the research answered the following objectives:

1) Whether the Filariasis CELISA could be easily adaptable for field survey work;
2) Whether seroprevalence mapping, using the Filariasis CELISA, could identify potential foci;
3) Once identified, how antibody serology could complement the already WHO approved diagnostic tools and whether antibody serology is superior to current diagnostic methods for defining an interruption of transmission;

4) The feasibility of the current protocols for control and elimination in ***Ae. polynesiensis*** vector endemic areas. In addition, whether there is spatial clustering of LF and, if so, if there is a spatial relationship to ongoing exposure in children; and,
5) Why there are systematically non-compliant individuals residing in these areas of residual foci.

The findings from this research are important for LF programme managers in order to aid successful global elimination of this debilitating disease.

CHAPTER 2

LITERATURE REVIEW

2.1 AETIOLOGY AND EPIDEMIOLOGY

LF is a mosquito-transmitted parasitic disease caused by the filarial nematodes ***W. bancrofti***, ***B. malayi,*** and ***B. timori*** (Ottesen, 2006). Bancroftian filariasis, due to the parasite ***W. bancrofti***, occurs throughout the tropics and some sub-tropical regions, and constitutes the majority of filariasis cases (90%). ***B. malayi*** is responsible for about 8% of cases and is confined to Asia. Lastly, ***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) (Figure 2.1). LF, even today, continues to be a major cause of morbidity worldwide with currently 128 million people infected and estimates of a further 1.3 billion people at risk of exposure (WHO, 2009). Of those infected, 40 million people face socioeconomic loss due to incapacitation and disfigurement (WHO, 2005). Consequently, this debilitating disease is ranked by the WHO as the second leading cause of permanent disability worldwide, after mental illness (Mathieu ***et al.***, 2008).

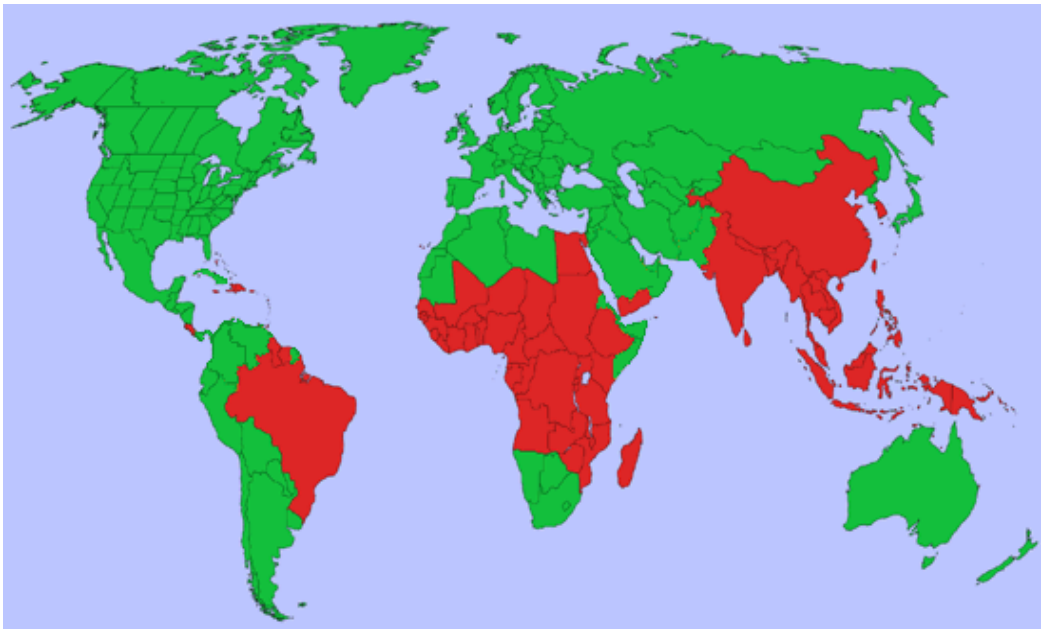


Figure 2.1: The geographical distribution of LF. The majority of cases are caused by the filarial nematode ***W. bancrofti*** (Melrose, 2004).

In 1997, the 50th WHA approved a resolution calling for the elimination of LF as a public health problem (WHA50.29) (WHO, 2005). The resolution acknowledged the morbidity and socioeconomic costs of LF including the general lack of awareness of disease and the potential for its eradication (CDC, 1993). The GPELF was developed in 1999 based on a comprehensive strategy to eliminate LF as a public health problem by the year 2020 (WHO, 2005). This included the interruption of transmission at a community-wide level and, for those already afflicted, individual patient management for morbidity control (WHO, 2000). This goal appeared feasible since two major drug companies, GlaxoSmithKline (GSK) and Merck, Sharpe & Dohme Incorporated generously volunteered to donate the medication necessary for treatment (Gustavsen ***et al.***, 2009).

There is a distinction between the prevalence of infection in the population and the intensity of infection (Mf numbers in the human host). Typically, prevalence and intensity are correlated, since when there is low intensity (low Mf numbers in host) there is less transmission and prevalence declines. However, this can be complicated by some infected individuals harbouring high density of Mf (Mathieu ***et al.***, 2006; El-Setouhy ***et al.***, 2007). These individuals potentially contribute to ongoing transmission (Mathieu ***et al.***, 2006; El-Setouhy ***et al.***, 2007). As there is a correlation between intensity of infection in the human populations and prevalence in the community, and prevalence is more easily determined, this thesis uses low prevalence as a proxy for low intensity (Sasa and Mitsui, 1964; Sasa, 1976).

Transmission intensity of LF is not only a product of the prevalence and density of Mf in the human host, but also the vector capacity of the mosquito (Southgate, 1992). As a biological vector, the mosquito is involved in the lifecycle of the parasite and each species differs in its ability to support maturation of ingested Mf to infective larvae stage three (iL3) (Southgate and Bryan, 1992; Snow ***et al.***, 2006; Bockarie ***et al.***, 2009a). ***Culex***, ***Anopheles***, ***Mansonia***, ***Ochlerotatus*** and ***Aedes*** (most commonly ***Ae. polynesiensis***) are the five genera of mosquitoes that serve as vectors for the parasite (Bockarie ***et al.***, 2009a; Manguin ***et al.***, 2010). Vectors can demonstrate either "facilitation" or "limitation" (Southgate and Bryan, 1992; Snow ***et al.***, 2006). ***Anopheles*** sp. are facilitators resulting in a positive correlation between the density of Mf ingested and the ability of the vector to transmit iL3 (Southgate and Bryan, 1992). Therefore, it is conceivable that, as intensity declines due

to treatment (Mf numbers in the human host), the vector becomes less effective in transmitting iL3, thus accelerating the decline in infection prevalence.

Ae. polynesiensis and ***Culex*** sp. display the characteristic of limitation (Snow ***et al.***, 2006; Pedersen ***et al.***, 2009); the proportion of Mf ingested which develop successfully to iL3 increases as the Mf density decreases (Pichon, 2002). This has a potential impact on LF elimination in countries where these vectors are present; transmission may persist at low parasite levels, leading to resurgence as has been previously recorded (Section 2.6.2) (Failloux ***et al.***, 1995). This can potentially reverse the degree of prevalence of infection in the community. Consequently, in ***Ae. polynesiensis*** or ***Culex*** sp. endemic areas, stringent surveillance is required to detect any threats of resurgence.

Peak levels of circulating ***W. bancrofti*** Mf in the bloodstream of the host vary among LF endemic regions depending on the biting habits of the local mosquito vector (Manguin ***et al.***, 2010). In the nocturnally periodic form, Mf appear in the blood during the night, usually peaking after 2200 hours, to be ingested by the nocturnal biting ***Anopheles*** sp. or ***Culex*** sp. (Manguin ***et al.***, 2010). In the diurnal sub-periodic form, found in areas where ***Aedes*** sp. is the vector, Mf appear in the blood during the day (Manguin ***et al.***, 2010).

2.1.1 Aetiology in the South Pacific

Endemnicity of LF in the South Pacific was documented as early as 1785, when Captain James Cook noted elephantiasis in Tonga (PacELF, 2006). Elimination of LF in the South Pacific poses many challenges (reviewed in Huppatz ***et al.***, (2008)). These include geographical remoteness, funding, baseline prevalence of LF, vectors present, threat of resurgence, and MDA compliance (Chanteau ***et al.***, 1995; Esterre ***et al.***, 2001; Ichimori and Crump, 2005; Huppatz ***et al.***, 2008; Kyelem ***et al.***, 2008).

Under the auspices of the GPELF, PacELF was formed with the initiative of eliminating Bancroftian LF as a public health problem by 2010, including morbidity management (PacELF, 2006; Ichimori ***et al.***, 2007a). In the Pacific, the majority of transmission is with the aedine (predominantly ***Ae. polynesiensis*** and anopheline species. Vector control, as part of the elimination strategies, was only thought to be feasible in areas where the aedine species did not occur. The longitude line of 170^{o} east marks the approximate transition zone for the vectors in the South Pacific (Manguin ***et al.***, 2010). West of the line consists mainly of the anopheline vectors whereas east is exclusively the aedine species (Manguin ***et al.***, 2010). Where the aedine vector is endemic, LF is particularly difficult to control (Kyelem ***et al.***, 2008), and these areas are associated with resurgence (Chanteau ***et al.***, 1995) because the efficiency of the vector increases as Mf prevalence declines (Samarawickrema ***et al.***, 1985a).

Vector control strategies in anopheline endemic areas rely predominantly on residual spraying of insecticide indoors and/or use of bednets (with or without insecticide treatment) (Bogh ***et al.***, 1998; Bockarie ***et al.***, 2002). More recently, odour-baited containers have shown promise for trapping and killing anopheline species (Okumu ***et al.***, 2010). Due to the day-biting tendencies of ***Ae. polynesiensis***, vector control strategies are thought to be challenging since traditional interventions used in areas of nocturnal periodicity, such as bednets (Burkot ***et al.***, 2002) and control of breeding sites around the household, are thought not to be effective (Bockarie ***et al.***, 2009a). Secondly, common breeding sites were thought to be restricted to coastal areas, such as crabholes found in shorelines (Rakai ***et al.***, 1974). However, other known breeding sites of the vector include anywhere that rainfall will collect such as discarded tins, drums, tree holes, canoes, and even in the leaves of the ***Pandanus*** (Bockarie ***et al.***, 2009a). Recently, it was demonstrated that the ***Aedes*** sp. had an affinity for both natural and man-made containers and, in the dry season, containers associated with households were significantly more likely to contain mosquito pupae (Lambdin ***et al.***, 2009). The authors concluded that because in ***Ae. polynesiensis*** endemic areas a spatial relationship between infected individuals has not been established, vector control strategies were unlikely to be feasible since source reduction campaigns would need to target entire villages (Lambdin ***et al.***, 2009; Mladonicky ***et al.***, 2009). I challenge this view, since biting tendencies of the major vector peak just after sunrise until approximately 0800 hours and again before sunset (Jachowski, 1954; Ramalingam, 1968; Rakai ***et al.***, 1974; Bockarie ***et al.***, 2009a), when individuals are most likely to be still in and

around their homes. This hypothesis is researched as part of my thesis (Chapter 7).

2.1.2 Lifecycle of *Wuchereria bancrofti*

The lifecycle of the parasite which causes LF occurs in both human and female mosquito hosts (Figure 2.2). Mf circulate in the blood of humans at night or day, depending on vector endemnicity (Sack, 2009; Manguin ***et al.***, 2010). It has been hypothesised that this phenomenon is linked to host melatonin levels signalling the solar dark/light cycle (Sack, 2009). Mf ingested by a susceptible mosquito penetrate the gut wall, migrate through the haemocele, and undergo maturation in the thoracic muscles to iL3, taking two to four weeks (Sasa ***et al.***, 1976; Christensen and Sutherland, 1984; Agudelo-Silva and Spielman, 1985; Perrone and Spielman, 1986; Nayar and Knight, 1995; Bockarie ***et al.***, 2009b). Ingesting high numbers of Mf can kill the mosquito because of the physical disruption of gut-wall integrity (Jordan, 1959; Beerntsen ***et al.***, 1996) and the migration of iL3s out of the thoracic flight muscles (Lindsay and Denham, 1986). Following maturation, iL3s migrate to the proboscis and, during the next blood meal from a human host, enter the punctured skin (Murthy and Sen, 1981), and travel to the lymphatics where they mature to the adult stage of the worm. Typically this occurs in the afferent lymphatics and can take up to 12 months, during which time a person may be infected, but amicrofilaraemic with no detectable CFA (Sasa ***et al.***, 1976; Bennuru and Nutman, 2009a). The length of time until CFA is detectable in infected individuals is unknown now that LF prevalence is

declining (Lammie ***et al***., 1998). Male and female adult worms mate and the female worm produces Mf, which enter the bloodstream, this takes approximately 12 months (Sasa ***et al***., 1976). The Mf are then ingested by the mosquito during the next blood meal.

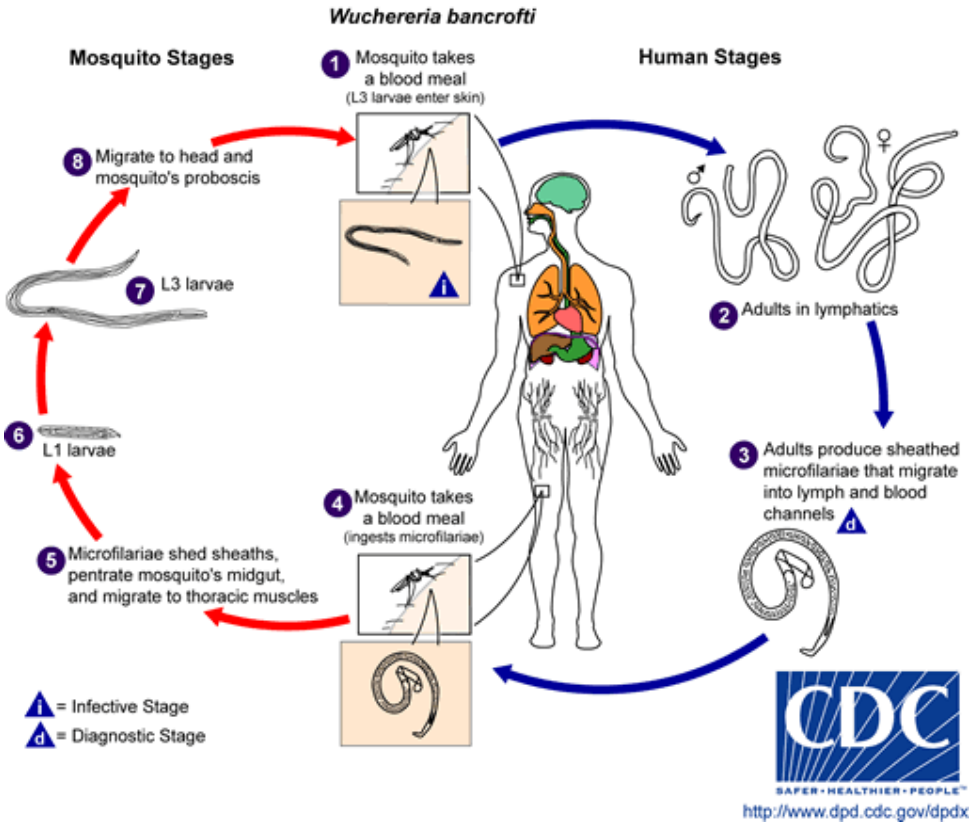


Figure 2.2: The lifecycle of *W. bancrofti*. Lifecycle occurs in both a human host and the intermediate female mosquito host. ***Sourced from Centers for Disease Control and Prevention (CDC) http://www.dpd.cdc.gov*** (CDC)

The ability of Mf to be ingested, undergo maturation, and infect a new host is not only dependent on the genera of mosquito (Section 2.1), but also the density of Mf in the host's peripheral blood, length of exposure to infective mosquitoes, and frequency of biting (Snow and Michael, 2002). For effective

numbers of iL3 to establish within the host, it has been estimated that the host needs to be bitten thousands of times (Hairston and de Meillon, 1968; Southgate, 1984; Ichimori and Crump, 2005). The magnitude of bites required, coupled with the relative inefficiency of transmission from the mosquito when compared with other mosquito-transmitted diseases such as malaria (Punkosdy ***et al.***, 2003; Ichimori and Crump, 2005), led to the consensus that LF would be an easily eradicable disease (CDC, 1993). This was also because of its reported successful elimination from Japan, Australia, Solomon Islands and Togo (Webber, 1975; Scheiber and Braun-Munzinger, 1976; Webber, 1977; Webber, 1979; Webber and Southgate, 1981; Boreham and Marks, 1986; Webber, 1991; Ichimori ***et al.***, 2007a). However, there is strong evidence that the task will not be easy in some endemic areas, especially in some of the Pacific Island Countries and Territories (PICT), where apparently successful efforts have been followed by resurgence (Section 2.6.2). I also have strong concerns that the "one size fits all" approach to the design and implementation of the WHO-sponsored GPELF may not be appropriate for all endemic areas, especially the PICT, because of the heterogeneity of LF transmission (Gambhir ***et al.***, 2010).

Consequently the following questions need answering:

1) Are the current thresholds for confirming absence of transmission adequate, or do areas with different epidemiology and vectors require different thresholds?
2) Are the current diagnostic tools adequate in a low prevalence, low parasite density environment, to detect residual foci of

active transmission (so-called “hotspots”), or confirm the absence of transmission, or provide an adequate surveillance system to detect early resurgence?

3) How big a threat does systematic non-compliance to MDA pose to elimination efforts (those who have never participated in MDAs (Talbot ***et al.***, 2008))? Who are these non-compliers, why are they non-compliant, and what can be done to improve compliance? The word “systematic” is used in the LF program to define those individuals who are consistently non-compliant with drug administrations (El-Setouhy ***et al***., 2007).

I believe that these questions are extremely important and must be urgently addressed if the GPELF is to be successful.

2.1.3 Elimination of Lymphatic Filariasis as a Public Health Problem in the South Pacific

Originally, PacELF had set the target of LF elimination as a public health problem by 2010 (PacELF, 2006; Ichimori ***et al.***, 2007a). The strategic plan focussed on the scheduling of a minimum of five rounds of MDA, depending on baseline prevalence in each country and results of post-MDA surveys (PacELF, 2006). Since then, prevalence surveys from 2007 highlighted the need to intensify efforts together with the recognition for more sensitive diagnostic assays (Chanteau and Roux, 2008). With the deadline fast approaching, and most scheduled MDAs already completed, it is imperative

to monitor the effectiveness of the treatment. This is especially due to reports of ongoing high prevalence in certain clusters of islands, such as Samoa (PacELF, 2005; Huppatz ***et al.***, 2009). Subsequently, the review committee which oversees annual reports, referred to as the PacELF Coordination and Review Group (PacCARE), solicited the help of an external consultant to aid the drafting of a surveillance strategy; the "LF surveillance strategy for the PICT" (WHO, 2007a), which is further discussed in Section 2.6.

The ongoing high prevalence of LF in certain endemic clusters could be due to a number of reasons (Huppatz ***et al.***, 2009). Although the disease was thought to be easily eliminated, difficulties have arisen as research uncovered more information about the disease. The ability of the parasite to survive within the human host using immunosuppression mechanisms (Hoerauf ***et al.***, 2005), coupled with the apparent lack of MDA compliance (Vaishnav and Patel, 2006; El-Setouhy ***et al.***, 2007; Talbot ***et al.***, 2008), has impeded LF elimination in some endemic areas (Kyelem ***et al.***, 2008). Endemic areas where these problems occur need to be identified in order to target PacELF's efforts for LF elimination. Non-compliant individuals could potentially remain as reservoirs and, thus, contribute to ongoing transmission (Mathieu ***et al.***, 2006).

To demonstrate complete elimination it is important to apply the correct LF diagnostic tools for this final phase, as well as implement successful

surveillance systems in endemic countries to monitor potential future outbreaks (Weil and Ramzy, 2007). This would allow immediate effective control of the outbreak areas to minimise impact and quickly reduce transmission below the threshold. Insensitive diagnostic assays in this final stage would underestimate prevalence or fail to detect residual areas of foci and potentially be responsible for reported resurgences in the past (Section 2.6.2). I hypothesise that antibody serology will be the diagnostic tool of choice, providing earlier warning of resurgence or identifying areas of residual endemnicity by assaying exposure rather than infection.

This thesis concentrates on application of the new anti-filarial antibody assay, the Filariasis CELISA, as a means for future surveillance. The literature review therefore outlines all diagnostic tools available, their limitations and advantages, and their application for declaring countries LF-free now and into the foreseeable future.

2.2 CLINICAL PRESENTATION

2.2.1 Introduction

LF has a wide spectrum of clinical manifestations because of the complicated immunopathology of the disease (Das ***et al.***, 1996; Bennuru and Nutman, 2009b). Lymphatic dysfunction occurs in filarial infected individuals, symptomatic or asymptomatic, either due to inflammation and/or excreted or secreted parasite products and/or mechanical obstruction of the flow of

lymph (Section 2.3) (Chewoolkar and Bachile, 2008; Bennuru and Nutman, 2009b; Khan ***et al.***, 2010). Based on current global estimates, 12.5% of all filarial infected people will suffer lymphoedema and 20.8% will suffer hydrocoele (Ottesen ***et al.***, 2008).

It is generally agreed that there are five broad categories of presentation in endemic areas (Dasgupta ***et al.***, 1984; Partono, 1987; Ottesen and Nutman, 1992; Evans ***et al.***, 1993; Njenga ***et al.***, 2007b):

- Endemic normals: those that have been exposed, but show no clinical symptoms and are Mf/CFA negative;
- Microfilaraemic asymptomatic;
- Acute filarial disease: Mf is either present or absent;
- Chronic disease: again with or without Mf;
- Tropical eosinophilia.

2.2.2 Asymptomatic Lymphatic Filariasis

Originally, the majority of these individuals were thought to be uninfected since there are no accompanying clinical symptoms (Weil ***et al.***, 1996). However, these individuals are often the most important group to identify for treatment because damage is still occurring (lymphangiectasia) and will eventually lead to symptomatic conditions (Suresh ***et al.***, 1997; Chewoolkar and Bachile, 2008). Once symptomatic, the individual can suffer from acute filarial attacks (Section 2.2.3.1) and, if left untreated, eventually progress to chronic disease (Section 2.2.3.2). Chronic stages of disease are usually

irreversible. Consequently, it is crucial to identify these individuals in the initial stages of infection to promptly administer treatment.

2.2.2.1 Endemic normals

The parasite-host relationship is a complex one, involving a balance between the density of the incoming iL3, the immunity of the individual, and other environmental factors such as vector distribution (reviewed in Melrose, (2004); Njenga ***et al.***, 2007b). Therefore, in some individuals living in endemic areas where exposure is frequent, infection may not be established if the immune system is robust in destroying incoming iL3 (Section 2.3.2). These individuals are referred to as "endemic normals" and have evidence of exposure, by testing positive by anti-filarial antibody assays, but have no other clinical evidence of infection (Weil ***et al.***, 1996).

As technology progressed, individuals originally classified as "endemic normals" were found to harbour adult worms in their lymphatics, but were asymptomatic (Dreyer ***et al.***, 1996a; Weil ***et al.***, 1996; Suresh ***et al.***, 1997). It has therefore been proposed that the term "endemic normals" should be divided into those truly uninfected and those that are infected, but asymptomatic and amicrofilaraemic, since a positive CFA test is a true indicator of infection (Weil ***et al.***, 1996; Weil ***et al.***, 1999).

The importance here is that while the individual remains unaware of infection, lymphatic damage is occurring in response to infection (Chewoolkar and

Bachile, 2008; Palumbo, 2008; Yuvaraj ***et al.***, 2008). Most importantly, it has been demonstrated in the Nile Delta region in Egypt, that 21% of these individuals could develop microfilaraemia within a year posing an infection risk to others (Weil ***et al.***, 1999). These Mf positive individuals, if still asymptomatic, become part of the group "asymptomatic microfilaraemics".

2.2.2.2 Asymptomatic microfilaraemics

Asymptomatic microfilaraemics are Mf positive, but display no clinical symptoms and, unless tested, do not realise they are infected. They represent the majority of the population in endemic areas and, sadly, in high LF prevalence areas children as young as 14 months were historically reported to be part of this group (Lowman, 1944). This group represents the highest threat to the LF programme since they are often left untreated, facilitating ongoing LF transmission (Mathieu ***et al.***, 2006; Bockarie ***et al.***, 2009b).

The progression to displaying clinical symptoms could take decades (Ottesen, 1992). However, in the meantime considerable damage to the lymphatics and renal system could be occurring, as observed with ultrasound studies (Freedman ***et al.***, 1994; Freedman ***et al.***, 1995; Dreyer ***et al.***, 1999a; Melrose, 2004; Shenoy ***et al.***, 2007; Chewoolkar and Bachile, 2008; Shenoy ***et al.***, 2008). Although the lack of clinical symptoms and absence of an inflammatory response in these individuals is quite remarkable because of

the ensuing damage from infection, they do require inclusion in any treatment programme.

2.2.3 Symptomatic Lymphatic Filariasis

The symptomatic categories of LF are acute and/or chronic. The acute form of the disease can be microfilaraemic or amicrofilaraemic, the amicrofilaraemic form is prone to misdiagnosis because the acute filarial attacks can mimic malaria (Melrose, 2004). Intermittent acute attacks can also occur in chronic LF (Dreyer ***et al.***, 1999b; McPherson ***et al.***, 2006).

2.2.3.1 Acute filarial disease

Acute filarial disease was originally thought to only manifest as adeno-lymphangitis (ADL), but it was discovered that there were two distinct clinical manifestations divided into acute filarial lymphangitis (AFL) and acute dermatolymphangioadenitis (ADLA) (Dreyer ***et al.***, 1999b). AFL, although relatively uncommon in untreated individuals, is caused by death of the adult worms and can be either asymptomatic or symptomatic (Dreyer ***et al.***, 1999b). ADLA is believed to be primarily caused by secondary bacterial infections because of the presence of skin lesions in these patients, which serve as a point of entry, and positive bacterial cultures from biopsies (Olszewski and Jamal, 1994; Shenoy ***et al.***, 1995; Dreyer ***et al.***, 1999b; Ananthakrishnan and Das, 2004). The skin lesions can be caused by fungal

infections, from improper hygiene, or minor traumatic injuries, including walking around barefoot (Dreyer ***et al.***, 2002a; Njenga ***et al.***, 2007b).

Clinically in males, AFL can manifest as orchitis, epididymitis and acute transient painful hydrocoele (Wartman, 1947; Nanduri and Kazura, 1989; Noroes ***et al.***, 2003; Noroes ***et al.***, 2009). Acute hydrocoele can develop into chronic hydrocoele (Section 2.2.3.2) (Noroes ***et al.***, 2003). In addition, men with LF progress to genital lymphoedema (Section 2.2.3.2). Females can suffer mastitis (Dreyer ***et al.***, 1999b; Ichimori and Crump, 2005). Biopsies of the inflamed nodules reveal a bolus of degenerating adult worms encased in a granuloma composed predominantly of eosinophils (Dreyer ***et al.***, 1999b). Unfortunately, the side effects of diethylcarbamazine citrate (DEC) treatment can mimic these attacks, because of the death of adult worms, thus raising issues with drug compliance (Dreyer ***et al.***, 1999b; Cantey ***et al.***, 2010). This is because, as the worm dies in response to the treatment, the secreted worm antigens can elicit an allergic reaction (Ottesen, 1984; Ottesen, 1987; Kar ***et al.***, 1993a; Dreyer ***et al.***, 1999b).

ADLA encompasses intense lymphangitis, lymphadenitis with retrograde extension from the affected node, and reddening of the overlying skin (Dreyer ***et al.***, 1999b; reviewed in Mendoza ***et al.***, (2009)). ADLA attacks can last for a week and are extremely debilitating, being accompanied by chills and fevers potentiated by the release of tumour necrosis factor alpha (TNF-α) from immune cells (Das ***et al.***, 1996). Rare sequelae include sepsis (Dreyer ***et al.***, 1999b). The acute attacks usually confine people to their houses, thus

economically burdening the afflicted families (Gyapong ***et al.***, 1996; Ramaiah ***et al.***, 1998; Dreyer ***et al.***, 1999b; Ramaiah ***et al.***, 2000a; Babu and Nayak, 2003).

Those who suffer from AFL have a higher risk of developing ADLA and, in turn, the frequency of ADLA attacks is correlated with increasing severity of the lymphoedema (Olszewski and Jamal, 1994; Pani ***et al.***, 1995; Shenoy ***et al.***, 1995; Dreyer ***et al.***, 1999b; Shenoy ***et al.***, 1999; Dreyer ***et al.***, 2000; Suma ***et al.***, 2002; Ananthakrishnan and Das, 2004; Yahathugoda ***et al.***, 2005; Palumbo, 2008). Those categorised with type 1 lymphoedema suffer the most frequent attacks and the incremental stages of lymphoedema progress to elephantiasis (Dreyer ***et al.***, 2002a; reviewed in Mendoza ***et al.***, (2009)). Consequently, untreated ADLA will eventually progress to chronic lymphoedema (elephantiasis). Sadly, those that have already suffered ADLA have a limited response to DEC treatment and will only benefit from preventing and/or treating secondary bacterial infections (Olszewski and Jamal, 1994; Dreyer ***et al.***, 2006a). These studies reiterate the importance of early LF treatment, recognition of interdigital skin lesions and appropriate management to prevent irreversible elephantiasis.

2.2.3.2 Chronic filarial disease

The well-known manifestation of LF is the progression to the chronic form of the disease, commonly referred to as elephantiasis, marked by chronic lymphoedema and/or hydrocoele (Ottesen, 2006; Kerketta ***et al.***, 2007). A

distinction must be made between the two clinical presentations; hydrocoele is the accumulation of serous fluid, commonly in the scrotal sack (Madan and Madan, 2007), whereas lymphoedema is the accumulation of lymph due to lymphatic obstruction either due to the worm or inflammation (Khan ***et al.***, 2010).

The first clinical symptom of progression to chronic disease is the gradual development of hydrocoele and/or lymphoedema, with or without Mf, following an acute attack (Evans ***et al.***, 1993; Michael ***et al.***, 1994; Shenoy, 2008). This process is a gradual one, often taking many years (Dreyer ***et al.***, 2002a; Nielsen ***et al.***, 2002a). The pathogenesis of chronic LF is controversial, since conflicting results have been published concerning the absence or presence of active LF in lymphoedema (Lammie ***et al.***, 1993; Estambale ***et al.***, 1994a; Addiss ***et al.***, 1995; Alexander, 2000; Simonsen ***et al.***, 2002; Kerketta ***et al.***, 2007; Njenga ***et al.***, 2007b). However, it is generally agreed that hydrocoele is often associated with active LF (Njenga ***et al.***, 2007b). Work is still being completed to determine if lymphatic damage is dominated by parasite mediated mechanisms or immune mediated mechanisms (Section 2.3). Either way, elephantiasis is the sequelae of repeated episodes of ADLA. In addition, foetal sensitisation to the parasite can play a role in increasing the affected child's risk of developing chronic pathology (Ottesen, 1992; Pani and Srividya, 1995; Dreyer ***et al.***, 2000; Dreyer ***et al.***, 2002b; Malhotra ***et al.***, 2006; Kerketta ***et al.***, 2007).

Recently, it has been demonstrated that filarial worms affect the cellular functions of the lymphatics and that these filarial-exposed lymphatics become unable to reabsorb interstitial fluid resulting in lymphoedema (Morchon ***et al.***, 2008). Following these findings, it was revealed that filarial lymphoedema was not parasite induced, but rather sequelae to the host inflammatory response, with accompanying fibrosis and cellular hyperplasia in and around the walls of the lymphatics (Bennuru and Nutman, 2009b). The complex immunopathology behind the disease is therefore yet to be clearly ascertained and most likely to be contributed to by both the parasite and the ensuing immune response.

Elephantiasis is the most debilitating form of LF, is often not treatable with medication, and is associated with severe socioeconomic loss and social stigma (Ramaiah ***et al.***, 1998; Ramaiah ***et al.***, 2000a; Babu ***et al.***, 2002; Suma ***et al.***, 2003; Person ***et al.***, 2007a; Person ***et al.***, 2007b; Person ***et al.***, 2008; Babu ***et al.***, 2009a). Treatment for the chronic stage relates to the management of lymphoedema (Kerketta ***et al.***, 2007) including ways to increase the flow of blood and lymph through the limb by elevation, massage, compression bandaging, and/or exercise, antibiotics to reduce the frequency of acute attacks, and proper foot/limb hygiene (Bingham, 2002; Dreyer ***et al.***, 2002a; WHO, 2004; Kerketta ***et al.***, 2007; Narahari ***et al.***, 2007; Njenga ***et al.***, 2007b; Shenoy, 2008; Wijesinghe ***et al.***, 2008). Although compression bandaging was originally recommended for basic lymphoedema management, in Haiti this was associated with an increased risk of ADLA, which was reduced when using basic hygiene and self-care (Addiss ***et al.***,

2010). Delays in treatment and inappropriate use of DEC during chronic lymphoedema have been documented (Wijesinghe ***et al.***, 2008).

Other treatments are surgical, including implantation of shunts (Chewoolkar and Bachile, 2008), lymphosuction or surgical excision of the oedematous subcutaneous tissues (Shenoy, 2008; Zacharakis ***et al.***, 2008). Hydrocoelectomy is often indicated for those with hydrocoele (Ahorlu ***et al.***, 2001; Njenga ***et al.***, 2007b). Surgical intervention is not often available to everyone (Njenga ***et al.***, 2007b), especially if there is poor nutritional status (Chewoolkar and Bachile, 2008), or patients are overlooked because of social stigma surrounding this chronic stage (Person ***et al.***, 2008). In some areas, sufferers can be ostracised from the village and often live alone in the outskirts (Person ***et al.***, 2008). On a recent field trip, one afflicted lady presented at the nurses station after nightfall, so as not to be seen by the other villagers (Hayley Joseph, ***unpublished observation***, 2008).

Debilitating elephantiasis is often complicated by secondary bacterial and fungal infections, the humid folds of the skin creating a niche for these organisms (reviewed in Mendoza ***et al.***, (2009)). This makes proper hygiene one of the most important treatments for individuals at this stage of disease, which unfortunately is often impossible in those areas without an adequate fresh water supply (Dreyer ***et al.***, 1999b; Kerketta ***et al.***, 2007). It is therefore in the individual's best interests to be tested and properly treated prior to the disease developing into this chronic stage. Secondly, it is not uncommon for those with chronic lymphoedema to also suffer intermittent acute episodes (Kumaraswami, 2000; Kerketta ***et al.***, 2007). This again highlights the

importance of proper hygiene for reducing the chances of secondary bacterial infections.

2.2.3.3 Tropical eosinophilia

A rare sequelae to disease is a hypersensitivity reaction manifesting in the lungs of infected individuals, found in southern India (Ray ***et al.***, 1993) and parts of South East Asia (Melrose, 2004), referred to as tropical eosinophilia. The common features are a marked eosinophilia with high anti-filarial antibody titres of both Immunoglobulin (Ig) E and IgG (Ottesen ***et al.***, 1979; Melrose, 2004). Diagnostically these individuals may be amicrofilaraemic, but will have adult worms as detected by ultrasound and thus test positive for CFA (Tan ***et al.***, 1985; Dreyer ***et al.***, 1996b; Vijayan, 2007). Clinical symptoms are asthma-like with nocturnal coughing, and the main concern with disease is the long term damage due to the hyper-inflammatory response (Pinkston ***et al.***, 1987). Marked eosinophilia has also been observed post-treatment of LF patients with DEC alone (Ottesen and Weller, 1979).

2.2.4 Drug Regimes

The regime of choice by PacELF to treat filarial disease is a combination of oral albendazole (400 mg), an anthelmintic, and DEC (6 mg/kg) (Ichimori and Crump, 2005; Ottesen, 2006). The beneficial effects of adding albendazole to the regime is controversial. Some studies suggest that the two-drug

combination enhances suppression of Mf in the blood to a greater extent than DEC alone (Addiss ***et al.***, 1997; Ismail ***et al.***, 1998; Molyneux and Zagaria, 2002). Other studies have failed to show a prolonged microfilaricidal or macrofilaricidal effect (Dreyer ***et al.***, 2006b). Despite the controversy, albendazole has collateral benefits since the prevalence of other important helminth parasites usually overlap LF (Hooper ***et al.***, 2009). Therefore, the obvious benefits of the inclusion of albendazole are thought to secure higher compliance rates (Padmasiri ***et al.***, 2006) as seen by the steady significant decline of soil transmitted helminthiasis infections in co-endemic areas (Rajendran ***et al.***, 2003; De Rochars ***et al.***, 2004; Mani ***et al.***, 2004; Oqueka ***et al.***, 2005; Ottesen ***et al.***, 2008).

DEC was the first anti-filarial drug and was discovered by Hewitt ***et al***. in 1947 (Oliver-Gonzalez ***et al.***, 1949). The primary mechanism of action of DEC is microfilaricidal via inhibition of arachidonic metabolism in Mf, making them susceptible to the host immune system (Maizels and Denham, 1992; Ottesen ***et al.***, 1997; McGarry ***et al.***, 2005). At clinical doses DEC has minor macrofilaricidal action as only approximately 50% of worm burdens are destroyed as observed by ultrasound studies (Ottesen, 1985; Noroes ***et al.***, 1997; Hussein ***et al.***, 2004; Kshirsagar ***et al.***, 2004). It is unknown whether there is a dose high enough which would be capable of killing all adult worms (Geary ***et al.***, 2009). However, a relatively higher macrofilaricidal activity has been reported when used in conjunction with albendazole (Kshirsagar ***et al.***, 2004); yet this remains controversial (Dreyer ***et al.***, 2006b). In addition, in areas of low prevalence, better performance of DEC alone has been reported

(Weil ***et al.***, 1999), possibly due to higher susceptibility of older worms to drug action (Mand ***et al.***, 2009). Despite its frequent and wide use, the drug is still highly effective today with little evidence of parasite resistance (Geary ***et al.***, 2009).

Unfortunately, parasite resistance against albendazole in animals has been recorded (Kaplan, 2004; Schwenkenbecher ***et al.***, 2007; Hodgkinson ***et al.***, 2008; Howell ***et al.***, 2008). This has led to controversy surrounding the overuse of albendazole since resistance in nematodes, intestinal parasites of humans, is thought to be imminent (Geary ***et al.***, 2009). This would have dire consequences for the treatment of helminthiases of humans in the future. In fact, it would render the treatment of these neglected tropical diseases (NTDs) challenging and be extremely detrimental to the LF programme, sparking the urgent need for other drug therapies (Geary ***et al.***, 2009). Advances in polymerase chain reaction (PCR) technology have allowed for the detection of albendazole resistance in nematode parasites of humans and, fortunately, none has yet been detected (Hoti ***et al.***, 2009). However, resistance is certain to emerge and it is important to continually monitor the population. Recently another anthelmintic compound, called Closantel, has been shown to be effective against onchocerciasis, via inhibition of moulting of larvae into iL3 (Gloeckner ***et al.***, 2010). This could also have potential for the LF programme.

The regime outlined by GPELF and PacELF included annual MDA rounds for four to six years, to reach at least 80% of the population, because of the long

term efficacy of the two-drug combination and mathematical modelling to interrupt transmission (WHO, 2000; Das ***et al.***, 2001; Ismail ***et al.***, 2001; Molyneux and Zagaria, 2002; Ramaiah and Das, 2004; Gyapong ***et al.***, 2005). The chosen number of rounds was linked to the lifecycle of the worm, since the estimated fecund lifecycle of the female adult worm is four to six years, but survival rates of up to 15 years in low prevalence settings have been observed (Vanamail ***et al.***, 1996; Dreyer ***et al.***, 2005; Ramaiah ***et al.***, 2009). Treatment was indicated annually since the approximate lifecycle of Mf is 12 months (reviewed in Bockarie ***et al.***, (2009b)). Mathematical modelling hypothesised that the number of rounds of MDA depended on the initial baseline LF prevalence and endemic vector species (Michael ***et al.***, 2004), which has also been demonstrated in the onchocerciasis control programme (OCP) (Diawara ***et al.***, 2009). Since the drugs used are highly microfilaricidal, by administering annual treatment, it was hypothesised that Mf would be suppressed and the incidence of infection would decrease (WHO, 2005). Efficacy of DEC therapy is dependent on the person's initial worm burden and their compliance with the medication, as persistence of antigenaemia can exceed 12 months if the initial dose is too low (Weil ***et al.***, 1988).

DEC is contraindicated in areas co-endemic for onchocerciasis because of the acute inflammatory response named the Mazzotti reaction (Stingl and Stingl, 1982; Francis ***et al.***, 1985). In these areas the drug regime of choice is ivermectin 200 to 400 µg/kg plus 400 mg of oral albendazole (Gyapong ***et al.***, 2005; Tisch ***et al.***, 2005). Ivermectin, although not used in the Pacific,

has also been shown to be microfilaricidal (Ottesen ***et al.***, 1990; Ottesen and Campbell, 1994). Unfortunately, ivermectin has no macrofilaricidal effects (Dreyer ***et al.***, 1996b). The pitfall of all investigations concerning worm burden following drug treatment is the long-term follow-up after drug administration. It is difficult to ascertain macrofilaricidal effects of drug action in areas of high transmission since no differentiation can be made between newly acquired adult worms and old adult worms.

Newer treatment regimes are currently being researched to target ***Wolbachia***, a bacterial endosymbiont necessary for filarial embryogenesis (Shakya ***et al.***, 2008; Specht ***et al.***, 2008; Supali ***et al.***, 2008; Bockarie ***et al.***, 2009b; Debrah ***et al.***, 2009; Foster ***et al.***, 2009; Hoerauf ***et al.***, 2009; Mand ***et al.***, 2009; Rao ***et al.***, 2009; Wu ***et al.***, 2009). These treatments render the female adult worm sterile (Hoerauf ***et al.***, 2000; Hoerauf ***et al.***, 2001; Hoerauf ***et al.***, 2003; Hoerauf ***et al.***, 2008) leading to eventual adult worm death (Debrah ***et al.***, 2006). The most common drug used to target ***Wolbachia*** has been the antibiotic doxycycline (Brouqui ***et al.***, 2001; Hoerauf ***et al.***, 2003; Taylor ***et al.***, 2005; Makunde ***et al.***, 2006; Turner ***et al.***, 2006; Debrah ***et al.***, 2007; Hoerauf ***et al.***, 2008; Korten ***et al.***, 2008; Mand ***et al.***, 2008; Supali ***et al.***, 2008; Hoerauf ***et al.***, 2009).

Targeting of ***Wolbachia*** using three week treatment regimes with doxycycline not only has beneficial effects in terms of macrofilaricidal activity, but also by its prevention or reversal of lymphatic pathology (Debrah ***et al.***, 2006; Debrah ***et al.***, 2009; Mand ***et al.***, 2009). The limitations of using doxycycline include

the need for daily treatment, side effects, and its contraindication in pregnant women or children under the age of 9 (Taylor ***et al.***, 2005; Bockarie ***et al.***, 2009b). This would not be feasible or sustainable for programmatic use because of the cost and potential for failure to comply with daily treatments (Bockarie ***et al.***, 2009b). Thus, new research is underway to identify potential targets of ***Wolbachia*** for drug therapy such as the heme biosynthesis pathway (reviewed in Bockarie ***et al.***, (2009b); Wu ***et al.***, 2009; Strübing ***et al.***, 2010). The potential role for doxycycline and other antibiotics, such as rifampicin, was recently reviewed by Bockarie ***et al***. (2009b). Although more research is required before the inclusion of anti-***Wolbachia*** treatment in WHO protocols, it is hoped that these treatment options will become available within the next few years (Bockarie ***et al.***, 2009b).

Encouragingly, globally, annual administration of five to six rounds of MDA has prevented 32 million disability-adjusted life years, positively impacting on the socioeconomic issues of LF, and 6.6 million neonates were protected from clinical disease (Ottesen ***et al.***, 2008; Hooper ***et al.***, 2009). This highlights the potential success of MDAs, when implemented efficiently. This is further evidenced by the successful elimination of LF as a public health problem in China and Korea, after the initiation of GPELF (WHO, 2008; Cheun ***et al.***, 2009a; Cheun ***et al.***, 2009b).

2.3 FILARIAL IMMUNITY

2.3.1 Introduction

Filarial immunity is a complex process involving a delicate balance between a predominantly T helper 2 (Th2) response with T helper 1 (Th1) assistance, since down-regulation of either response leads to parasite persistence (Elson ***et al.***, 1995; Dimock ***et al.***, 1996; Saeftel ***et al.***, 2001; Babu ***et al.***, 2006; Shakya ***et al.***, 2009; Vanam ***et al.***, 2009). It is believed this intricate balance holds the key for acquisition of protective immunity and any interruption results in the immunopathogenesis observed in acute and chronic filarial disease (reviewed in Melrose, (2004)). The actual mechanisms involved in protective immunity are still debatable and rely on a huge number of interplaying factors from host, parasite, and environment (Ottesen, 1980; Sahu ***et al.***, 2008). This includes, but is not limited to, intensity and duration of exposure, worm burden, level of endemnicity, lifecycle stage of the worm, immune status of patient, possible co-infection with other parasites, viruses, or bacteria, compliance with medication, age, gender, genetic predisposition and foetal sensitisation (Brabin, 1990; Kwan-Lim and Maizels, 1990; Day ***et al.***, 1991; Lammie ***et al.***, 1991; Kurniawan-Atmadja ***et al.***, 1998; Terhell ***et al.***, 2000a; Malhotra ***et al.***, 2003; Wahyuni ***et al.***, 2004; Nielsen ***et al.***, 2006; Jaoko ***et al.***, 2007; Cuenco ***et al.***, 2009; Dixit ***et al.***, 2009; Petersen ***et al.***, 2009; Wright ***et al.***, 2009). The summation of these effects means that the exact mechanisms involved in protective immunity are the subject of intense debate. This review attempts to briefly address the potential processes

involved in filarial immunity by concentrating specifically on humoral immunity. This focus is chosen because the Filariasis CELISA diagnostic assay detects anti-filarial IgG_4.

2.3.2 Innate Immune Response

During most parasitic infections the host relies primarily on a Th2 response, with appropriate humoral immunity, in order to contain and eliminate large extracellular parasites (Janeway ***et al.***, 2001). Humoral involvement includes secreted and excreted proteins such as cytokines, antibodies, and the proteins of the complement cascade (Janeway ***et al.***, 2001). Incoming iL3 encounter cells and components of the innate immune response, which aim to eliminate larvae using mechanisms such as the complement cascade (Janeway ***et al.***, 2001; Shakya ***et al.***, 2009). Complement cascade proteins function to directly lyse or opsonise filariae (Janeway ***et al.***, 2001). Opsonisation facilitates parasite recognition by cells such as macrophages and natural killer (NK) cells (Janeway ***et al.***, 2001).

Following recognition, receptors of NK cells bind to filariae, stimulating release of cytotoxic granules, which function to lyse filariae (Janeway ***et al.***, 2001). Th1-activated macrophages produce and release nitric oxide (NO) (Shakya ***et al.***, 2009), further activating NK cells (Bogdan ***et al.***, 2000). Macrophages also serve as antigen presenting cells (APCs) (Janeway ***et al.***, 2001). APCs recruit adaptive immune mechanisms if the innate immune response is unsuccessful in destroying the parasite (Janeway ***et al.***, 2001).

2.3.3 Adaptive Immune Response

2.3.3.1 Introduction

During the adaptive phase of the immune response in LF both Th1 and Th2 cytokine secretion has been observed (Le Goff ***et al.***, 2000; Le Goff ***et al.***, 2002; Babu ***et al.***, 2007a; Sahoo ***et al.***, 2009). The cocktail of cytokines determines whether a naïve T lymphocyte will switch to a Th1 or Th2 subset (Janeway ***et al.***, 2001).

Th1 lymphocytes play a role in aiding macrophage cellular activation, by release of Interleukin-2 (IL-2) and interferon gamma (IFN-g), enhancing internalisation of filarial antigen (Elson ***et al.***, 1995; Dimock ***et al.***, 1996; Saeftel ***et al.***, 2001; Babu ***et al.***, 2006; Shakya ***et al.***, 2009). Additionally, cellular activation aids passage of macrophages to lymph nodes, to recruit B lymphocytes via antigen presentation (Janeway ***et al.***, 2001).
B lymphocytes produce Ig directed against epitopes on the surface of the parasite or excreted products or secreted products (Kwan-Lim and Maizels, 1990). The role of each Ig isotype is discussed later (Section 2.3.3.2).

Th2 lymphocytes release cytokines such as IL-4 to stimulate B lymphocytes to produce and secrete Th2 antibodies including IgG_1, IgG_4, IgA, and IgE (Lundgren ***et al.***, 1989; Janeway ***et al.***, 2001; Brooker ***et al.***, 2004a). Other Th2 cytokines which play a central role in parasitic immunity include IL-5,

IL-9, IL-10 and IL-13 (Brooker ***et al.***, 2004a). This cytokine profile is consistent with the development of a strong IgE response (Janeway ***et al.***, 2001). Release of IL-5 at the site of infection also plays a role in the further recruitment of neutrophils, eosinophils and basophils (Janeway ***et al.***, 2001; MacDonald ***et al.***, 2002; Wright ***et al.***, 2009). Over-expression of Th2 cytokines is often associated with chronic infection, such as that which occurs in hookworm infections, and is a trademark for filarial tropical pulmonary eosinophilia (Brooker ***et al.***, 2004a; Vijayan, 2007). Over-expression of Th1 cytokines can also be associated with immunopathology, highlighting the intricate balance required for effective immunity.

A Th1-shift is associated with filarial pathology because of heightened inflammation, possibly in response to bacterial antigens from the endosymbiotic ***Wolbachia***, which has been observed in onchocerciasis (Punkosdy ***et al.***, 2003; Tsillassie and Legesse, 2007). Inflammation can occur upon worm death, since ***Wolbachia*** bacteria are located inside the adult worm (Cross ***et al.***, 2001; Egyed ***et al.***, 2002). Preliminary studies hypothesised that this inflammatory reaction against ***Wolbachia*** was directed against the bacterial lipopolysaccharide (LPS) (Taylor ***et al.***, 2000; Taylor ***et al.***, 2001). On the contrary, genome sequencing revealed that ***B. malayi Wolbachia*** does not encode the necessary enzymes for LPS synthesis (Foster ***et al.***, 2005), suggesting other mediators are involved, possibly including filarial surface proteins (Brattig ***et al.***, 2004; Porksakorn ***et al.***, 2007).

Wolbachia is not the only potential means for evoking a predominantly inflammatory response. Acute filarial attacks are believed to be triggered by parturition by adult worms, releasing CFA into the circulation, in turn causing periodic febrile episodes (Kar ***et al.***, 1993b). Whichever the means for the Th1/Th2 shift to immunopathology, the breakdown in the delicate balance contributes to the different clinical manifestations of disease.

2.3.3.2 Humoral Immunity

The isotype and titre of antibody produced plays an imperative role in protection against nematode infection (Hussain ***et al.***, 1987; Weil ***et al.***, 1992; Blackwell and Else, 2001; Janeway ***et al.***, 2001; Vedi ***et al.***, 2008; Shakya ***et al.***, 2009). During initial exposure, IgM antibodies against iL3 are important prior to isotype switching (Ottesen ***et al***., 1982; Kwan-Lim and Maizels, 1990; Hjelm ***et al.***, 2006) and function independently of T lymphocyte stimulation (Hjelm ***et al.***, 2006). Early studies have demonstrated the role of IgM in Mf clearance (Thompson ***et al.***, 1979; Storey ***et al.***, 1987) and activation of the complement cascade (Janeway ***et al.***, 2001; Hjelm ***et al.***, 2006). By binding to complement proteins, IgM forms antibody-antigen-complement complexes, which then cross-link B lymphocyte receptors (Janeway ***et al.***, 2001). Cross-linkage results in B lymphocyte stimulation, activation, isotype switching accompanied by production of other Ig, and antigen presentation to T lymphocytes (Hjelm ***et al.***, 2006). As the role of IgM is limited, other isotypes also contribute to filarial immunity.

The main function of anti-filarial antibodies, by binding to surface epitopes, is antibody dependent cellular cytotoxicity (ADCC) (Mehta ***et al.***, 1981; Kwan-Lim and Maizels, 1990; MacDonald ***et al.***, 2002; Vanam ***et al.***, 2009). ADCC is the mechanism by which foreign targets are coated with specific antibody, dependent mainly on the elevated cytophilic antibodies IgG_1 and IgE (Janeway ***et al.***, 2001). Once opsonised, the foreign target is recognised by other cells of the immune response, such as eosinophils and NK cells, which bind to the constant region of the antibody via specific cellular receptors (Janeway ***et al.***, 2001). These cells are attracted to the infected area by chemokines, such as IL-5 (MacDonald ***et al.***, 2002). Receptor binding, between the constant region of the antibody and the cell, activates lysis of the antibody-coated target. This is achieved via the release of cytoplasmic granules containing perforin and granzymes from the activated cell (Janeway ***et al.***, 2001).

2.3.3.2.1 IgG_1

IgG_1 is produced prior to anti-filarial IgG_4 following filarial exposure (Kwan-Lim ***et al.***, 1990; Wamae ***et al.***, 1992; Lammie ***et al.***, 1998; Wamae ***et al.***, 1998; Washington ***et al.***, 2004). The main role of IgG_1 in fighting filarial infection is via ADCC and it is unable to activate complement (Hjelm ***et al.***, 2006). One of the target epitopes of IgG_1, primarily in children, is phosphorylcholine (PC) (Hamilton, 1987; Kwan-Lim and Maizels, 1990). PC epitopes are commonly present in nematodes, bacteria and fungi (Leon and Young, 1971; Gutman and Mitchell, 1977; Young and Leon, 1977; Almond

and Parkhouse, 1986; Lal and Ottesen, 1989; van Riet ***et al.***, 2006; Kooyman ***et al.***, 2009). Molecules with PC are secreted by adult parasites and may play a role in immunosuppression (Mitchell ***et al.***, 1976; Weiss, 1985; Lal ***et al.***, 1990), as observed in the laboratory through suppression of mitogen-induced T lymphocyte proliferation (Wadee ***et al.***, 1987; Lal ***et al.***, 1990). Thus IgG_1 may play a role in blocking the immunosuppressive effect of PC, or coating the foreign molecule for ADCC.

Conflicting results have been published concerning the protective role of IgG_1 during filarial infection (Vedi ***et al.***, 2008; Shakya ***et al.***, 2009). As a predominantly Th2 Ig, increase in titre following vaccination with a 73 kilodalton (kDa) protein has been shown to give partial protective immunity in the jird model (Vedi ***et al.***, 2008). However, when using deoxyribonucleic acid (DNA) vaccine cocktails, low IgG_1 responses were associated with protection (Shakya ***et al.***, 2009). In the former study there was also evidence of elevated Th1-type IgG_2 isotype, which may have balanced the elevated Th2-type IgG_1 response (Janeway ***et al.***, 2001; Vedi ***et al.***, 2008). Due to the contradiction in results, further studies are required to validate the protective role of IgG_1. These studies could include specific isotype depletion studies in the jird model.

2.3.3.2.2 IgG_2

Roles of this Th1-type Ig include weak complement and monocyte binding (Janeway ***et al.***, 2001), and one of the target binding sites in adults is PC or

Mf sheath (Hamilton, 1987; Scott ***et al.***, 1987; Simonsen and Meyrowitsch, 1998). The protective role of IgG_2 is not yet fully understood, but preliminary studies demonstrated that elevated levels were similar between asymptomatic microfilaraemics and those with chronic pathology (Hussain ***et al.***, 1987). Conflicting studies showed that an individual was five-fold more likely to be amicrofilaraemic if they were anti-filarial IgG_2 positive (Hitch ***et al.***, 1991). These results were confirmed in later studies, whereby an inverse relationship between IgG_2 levels and CFA was observed (Simonsen and Meyrowitsch, 1998). The question arising from the research is whether elevated IgG_2 is associated with chronic pathology or infection or immunity. Further research is required.

2.3.3.2.3 IgG_3

Significantly higher levels of IgG_3, compared to the other IgG isotypes, have been found in elephantiasis and chronic pathology, possibly marking a relationship between this isotype and limb pathology (Hussain ***et al.***, 1987; Nielsen ***et al.***, 2002b; Wongkamchai ***et al.***, 2006). Lower levels of IgG_3 are observed during microfilaraemia (Hitch ***et al.***, 1991). The immune role of IgG_3 has been shown to be ADCC and complement activation (Janeway ***et al.***, 2001; Hjelm ***et al.***, 2006). As sequelae to excessive complement activation, the ensuing inflammation in the surrounding tissue could potentially have a damaging effect on the lymphatic vessels, leading to limb pathology (Nielsen ***et al.***, 2002b). Furthermore, the formation of immune complexes between antibody and antigen could be detrimental (Ottesen,

1980; Hjelm ***et al.***, 2006). The exact role of IgG_3 in filarial immunity is unclear, but appears to be related to CFA negativity, which does occur during chronic pathology (Jaoko ***et al.***, 2006).

2.3.3.2.4 IgG_4

Although in healthy individuals this subtype only makes up about 4% of total serum Ig (Janeway ***et al.***, 2001), IgG_4 is the dominant subclass produced during active filarial infection, either in asymptomatic or symptomatic individuals (Ottesen ***et al.***, 1985; Bal and Das, 1999). Rising levels of IgG_4, in both Brugian and Bancroftian filariasis, are associated with late exposure (persistent repeated exposure), filarial infection and microfilaraemia (Ottesen ***et al.***, 1985; Hussain ***et al.***, 1987; Kwan-Lim ***et al.***, 1990; Hitch ***et al.***, 1991; Estambale ***et al.***, 1994b; Mohanty ***et al.***, 2007). The isotype has been shown to be both beneficial and detrimental to the host, depending on titre and ratio to IgE (Hussain and Ottesen, 1985). The beneficial roles of IgG_4 are its inhibitory effects, primarily directed against IgE or filarial proteases (Hussain and Ottesen, 1986; Hussain and Ottesen, 1988; Satoh ***et al.***, 1999; Janeway ***et al.***, 2001; Bal ***et al.***, 2003; Hjelm ***et al.***, 2006; Mohanty ***et al.***, 2007).

Elevated levels of IgG_4, co-expressed with IgE, serve to block IgE effects via binding to the same epitope on the antigen surface (Hussain and Ottesen, 1986; Hussain and Ottesen, 1988; Satoh ***et al.***, 1999; Janeway ***et al.***, 2001; Mohanty ***et al.***, 2007). The parallel recognition serves as competition for the antigen and, by IgG_4 masking the antigen, prevents binding by other

B lymphocytes or antibodies (Hussain and Ottesen, 1988; Hjelm ***et al.***, 2006). This protective role serves to down regulate IgE-mediated effects on tissue pathology and results in an anti-asthmatic hyposensitisation (Satoh ***et al.***, 1999; Brooker ***et al.***, 2004a). This can be beneficial in blocking the severe IgE-mediated allergic responses such as anaphylactic shock and extensive tissue pathology (Ottesen ***et al.***, 1981a; Hussain and Ottesen, 1986; MacDonald ***et al.***, 2002; Mohanty ***et al.***, 2007). Rising levels of IgG_4 are therefore inversely proportional to levels of IgE (Maizels ***et al.***, 1995). For optimal clinical outcome the ratio of IgG_4 to IgE is crucial since excessively high ratios have been associated with microfilaraemia and the opposite true for tropical pulmonary eosinophilia (Hussain and Ottesen, 1985). In strongyloidiasis, it has been demonstrated that patients with high parasite-specific IgG_4 have decreased efficacy of treatment and were unable to be cured (Satoh ***et al.***, 1999). The opposite holds true for excessive IgE production since correlating studies demonstrated that antigen recognition patterns for IgG_4 were minimal during the stages of chronic pathology (Hussain ***et al.***, 1987). Chronic pathology may be associated with the over production of allergic mediators and inflammation. This reiterates the importance of an intricate balance among the immune elements for successful elimination of the parasite. Therefore, the balance is crucial in order to maximise the advantageous effect of IgE, which is necessary for elimination of the parasite (Janeway ***et al.***, 2001).

Another pathological mechanism by which filarial parasites are able to survive within a host is via secretion of filarial proteases, which serve to aid

tissue invasion, feeding, and embryogenesis (Pokharel ***et al.***, 2006). In onchocerciasis, filarial proteases have been shown to be crucial for parasite survival by enzymatically destroying connective tissue (Petralanda and Piessens, 1994). Anti-protease IgG_4 antibodies have been shown to dominate in asymptomatic carriers of LF and specifically target filarial protease (Bal and Das, 1999; Bal ***et al.***, 2003). Consequently, anti-filarial IgG_4 could potentially interfere with parasite survival.

2.3.3.2.5 IgE

The humoral immune response to filariasis is marked by production of IgE including both parasite-specific and non-specific polyclonal activation of synthesis of IgE (Jarrett and Bazin, 1974; Wahyuni ***et al.***, 2003). IgE production is crucial for the elimination of parasites (Brooker ***et al.***, 2004a) and results in cellular degranulation of the granulocytic cells; mast cell, basophil, and eosinophil via receptor binding (Janeway ***et al.***, 2001). Mast cell degranulation serves as a chemoattractant for eosinophils, thereby amplifying the response (Janeway ***et al.***, 2001). Cellular degranulation releases histamine and other mediators aimed to lyse and destroy the parasite (Janeway ***et al.***, 2001; Nielsen ***et al.***, 2002b; Brooker ***et al.***, 2004a). Studies have identified an inverse relationship between LF transmission and IgE levels, whereby areas of high endemnicity had reduced levels of IgE (Terhell ***et al.***, 2002). Functional activity of IgE is dependent on receptor interaction with target cells and the particular receptor can lead to either protective immunity or immunopathology (Wahyuni ***et al.***, 2003).

Excessive production of IgE results in serious allergic reactions, including anaphylactic shock (Lowenstein and Michel, 2006), and tropical pulmonary eosinophilia, where IgE levels were significantly higher than other clinical presentations of filariasis (Hussain ***et al.***, 1981). Consequently, high levels of IgE relative to IgG_4 have been positively associated with development of pathology (Nielsen ***et al.***, 2002b).

2.3.3.2.6 IgA

The protective role of IgA has been studied in other helminth infections and, more recently, Bancroftian filariasis (Lloyd and Soulsby, 1978; Grezel ***et al.***, 1993; Grzych ***et al.***, 1993; McCoy ***et al.***, 2008; Sahu ***et al.***, 2008). It was discovered that higher levels of anti-filarial IgA antibodies were present in the serum of endemic uninfected individuals, and, conversely, lower levels of IgA in microfilaraemic persons (Sahu ***et al.***, 2008). It was speculated that this lower level was due to the impairment of Th1 responses in macrophages, especially inhibiting NO production, which has been reported to play a role in filarial infection using the mouse model (Rajan ***et al.***, 1996; Mukhopadhyay ***et al.***, 2002; Zhang ***et al.***, 2002). Decreased Th1 responses contribute to T lymphocyte hyporesponsiveness. Hyporesponsiveness in LF has been observed for decades (Ottesen ***et al.***, 1977). In addition, it was found that there were significantly lower amounts of anti-filarial IgA in the serum of males (Sahu ***et al.***, 2008), which may contribute to the observed male predisposition to infection (Bell ***et al.***, 1999; Terhell ***et al.***, 2000a; Ganley-Leal, 2005). Earlier studies, investigating the use of a immunoassay for

detection of serum IgA, agreed with these findings as detectable IgA was found in only 50% of microfilaraemic patients (Chanteau ***et al.***, 1992). Exactly how IgA is protective against filarial infection is yet to be ascertained.

2.3.4 Pre-natal Sensitisation

The repertoire of immune responses can be further complicated by the presence of pre-natal sensitisation (Steel ***et al.***, 1994). During gestation there is foetal priming of filarial-specific T lymphocytes to release cytokines of the Th2 variety as excretory or secretory products cross the placenta to induce partial tolerance (Malhotra ***et al.***, 1999; Malhotra ***et al.***, 2003). Children born to infected mothers have been shown to have filarial-specific IgE present in the cord blood resulting in pre-natal allergic sensitisation (Weil ***et al.***, 1983; King ***et al.***, 1998). Anti-filarial IgG_4 and IgE levels in children born to infected mothers are positively correlated with maternal serum levels (Terhell ***et al.***, 2002). The resultant sensitisation is thought to result in a skewed Th2 response in these children, significantly increasing their susceptibility to infection, irrespective of paternal infection status (Lammie ***et al.***, 1991; Meyrowitsch ***et al.***, 1995; Malhotra ***et al.***, 2003).

2.3.5 Co-infection and Vaccine Efficacy

The hyporesponsiveness and immunosuppression observed in filariasis has dire consequences for susceptibility to other infections. LF endemic areas are often endemic for other parasitic diseases, including hookworm and

malaria, as well as other important diseases such as tuberculosis and Human Immunodeficiency Virus (HIV) (Keiser ***et al.***, 2003; Gallagher ***et al.***, 2005; Nielsen ***et al.***, 2006; Utzinger and de Savigny, 2006; Nielsen ***et al.***, 2007; Hotez ***et al.***, 2008; Brooker and Clements, 2009; Petersen ***et al.***, 2009). HIV positive individuals have higher levels of anti-filarial IgG_3, which is associated with chronic pathology in LF (Hussain ***et al.***, 1987; Wongkamchai ***et al.***, 2006). The Th1 cells of these individuals also have higher numbers of cellular receptors, up-regulated during the ascension of the Th2 LF response, which facilitates and enhances HIV infection of these cell subtypes (Brooker ***et al.***, 2004a). Therefore, it is no surprise that there is a positive correlation between incidence of HIV and LF (Nielsen ***et al.***, 2006).

Interestingly, studies on co-infection with malaria are conflicting (reviewed in Muturi ***et al.***, (2008); Manguin ***et al.***, 2010). Although co-occurrence of malaria and filarial parasites in a single human has been reported, with prevalence ranging from 0.4% to 3.3% (Prasad ***et al.***, 1990; Ghosh and Yadav, 1995; Ravindran ***et al.***, 1998; Chadee ***et al.***, 2003; Muturi ***et al.***, 2006), some studies suggest that this correlation does not reach levels of significance (Nielsen ***et al.***, 2006). There is also disagreement concerning the effect this concomitant infection has on the host. Some studies suggest LF exacerbates the malarial immunopathology, whilst others report no effects whatsoever (Graham ***et al.***, 2005; Muturi ***et al.***, 2006). Recent studies using the mouse model suggest that IL-10 production, as a consequence of filarial infection with ***Litomosoides sigmodontis***, has a protective effect against murine cerebral malaria (Specht ***et al.***, 2010). The conflicting studies are of

no surprise since a spatial study analysing the prevalence of LF and malaria in West Africa found a significant inverse relationship (Kelly-Hope ***et al.***, 2006). That is, in areas of high LF prevalence there were low levels of malaria. This was speculated to be because of competition for the same vector species and has important consequences for control programmes (Kelly-Hope ***et al.***, 2006). This is because control of one disease may inadvertently affect the epidemiology of the other. Further studies are required to ascertain the immunopathology caused by co-infections with malaria as well as co-existence of these two diseases in the same endemic area.

Reactivation of latent tuberculosis, following filarial infection, has been reported to occur due to diminishment of the Th1 and pro-inflammatory cytokine response necessary for ***Mycobacterium tuberculosis*** elimination or sequestration (Babu ***et al.***, 2009b). Similar results are observed for co-infection of hookworm with ***M. tuberculosis*** and/or HIV, since the immune response to the hookworm infection is predominantly Th2 (Brooker ***et al.***, 2004a).

Besides facilitating co-infection, filariasis may also adversely affect vaccine efficacy. Vaccine strategies rely heavily on mounting the correct immune response following antigen stimulation (Janeway ***et al.***, 2001). If this response is impaired, then production of specific memory cells will be inadequate. Filariasis has been shown to impair tetanus-specific humoral and cellular responses (Nookala ***et al.***, 2004), which has also been reported

for onchocerciasis (Prost ***et al.***, 1983; Cooper ***et al.***, 1999). These studies highlight the need for pre-treatment of individuals in LF endemic areas with anti-filarial medication prior to vaccination, in order to provide protective immunity against these vaccine preparations.

2.4 LABORATORY DIAGNOSIS AND APPLICABILITY IN THE FIELD

2.4.1 Introduction

Diagnostic assays hold the key for monitoring the progress of LF elimination and, finally, demonstrating its successful elimination. It is imperative to choose the correct and most appropriate diagnostic tool for each stage of the LF programme so as not to underestimate prevalence and/or incidence.

The three main markers for LF diagnosis are antigenaemia, microfilaraemia and/or presence of anti-filarial antibodies. In the past diagnostic assays for LF were rather limited, but high prevalence outweighed the need for highly sensitive diagnostic assays. Now that the prevalence is declining, and MDA endpoints need to be defined, more sensitive assays are required and becoming available (Weil and Ramzy, 2007), including the Filariasis CELISA anti-filarial antibody assay. To be included in the LF programme, the Filariasis CELISA requires validation in the field setting, since assay efficacy with large sampling in low prevalence settings is unknown (Weil ***et al.***, 2010). Secondly, LF endemic countries often do not have sophisticated laboratories

and they rely on either field testing or collecting samples in an easy format for transportation to laboratories capable of testing.

It is still an important consideration for laboratory diagnosis of LF to be aware of the lifecycle of the parasite, and to also make diagnoses based on clinical symptoms. Individuals may be infected with adult worms, but test negative for both Mf and CFA. This is linked to the lifecycle, since it can take 6 to 12 months before the adult worm matures, and it is the CFA derived from the adult worm that is detected (Weil ***et al.***, 1997). Upon maturation the male and female worm mate to produce Mf, which migrate to the bloodstream. Consequently, a person will be amicrofilaraemic before this occurs.

The currently approved diagnostic tools for the LF programme are those that detect Mf or CFA positive individuals (WHO, 2005). The sensitivity of these diagnostic assays when prevalence is low is unknown (Grady ***et al.***, 2007; Weil and Ramzy, 2007; Molyneux, 2009). This gap in knowledge highlights the urgent need for new diagnostic methods, such as antibody serology since it becomes positive prior to either Mf or CFA (Lammie ***et al***., 1998; Grady ***et al***., 2007; Weil and Ramzy, 2007; Molyneux ***et al***., 2009). Consequently, it is imperative that more sensitive diagnostic methods are approved for use, such as the Filariasis CELISA. This requires investigation to ascertain the suitability of this standardised assay, which is the main focus of my thesis. The findings have a major impact on the drafting of future surveillance strategies.

2.4.2 Circulating Filarial Antigen (CFA) Assays

CFA assays are particularly useful for diagnosing:

1) Infected amicrofilaraemic individuals (who do not pose an infection risk to others); and,
2) Asymptomatic infected individuals.

The CFA tests are designed to detect adult worm products in the bloodstream, indicating the presence of living adult worms in the host and thus active infection (Hamilton, 1985; Weil ***et al.***, 1999). They are superior to Mf detection assays for sensitivity and ease (Moulia-Pelat ***et al.***, 1993; Turner ***et al.***, 1993; Lammie ***et al.***, 1994; Wamae ***et al.***, 1998; Weil ***et al.***, 1999). This is because samples can be collected at any time, unlike the nocturnal or diurnal variation observed for Mf testing (Turner and Edeson, 1957; Moulia-Pelat ***et al.***, 1993; Lammie ***et al.***, 1994; Wamae ***et al.***, 1998; Weil ***et al.***, 1999).

Unfortunately, CFA assays only exist for the more common ***W. bancrofti*** infections, leaving other methods to detect ***B. malayi*** and ***B. timori***. CFA detection tools available for Bancroftian filariasis are the NOW® Filariasis Immunochromatographic Test (ICT) and the ***Onchocerca gibsoni*** 4C3 (Og4C3) ELISA (Trop Bio, Pty. Ltd., Australia) (Turner ***et al.***, 1993; Weil ***et al.***, 1997). The former is based on specific monoclonal and polyclonal antibodies, and is readily useable in the field (Schuetz ***et al.***, 2000) and the latter is based on a monoclonal antibody raised against an ***O. gibsoni*** antigen with strong specificity for the ***W. bancrofti*** antigen (More and Copeman, 1990;

More and Copeman, 1991; Melrose, 2004). Presence of CFA, measured by ICT or Og4C3, may not be detectable when prevalence declines because of the limits of sensitivities of the aforementioned diagnostic tests (Chanteau ***et al.***, 1994; Rocha ***et al.***, 1996; Njenga ***et al.***, 2007a). Recently, an assay to detect CFA based on monoclonal antibodies raised against SXP-1 has been trialled (Janardhan ***et al.***, 2010). The authors claim this method to be more sensitive than existing assays testing for CFA (Janardhan ***et al.***, 2010). Despite this, the sensitivity of CFA testing when prevalence is low is unknown and, coupled with a lag phase of up to 12 months before an infected person tests positive, could be unsuitable for surveillance (Grady ***et al.***, 2007; Weil and Ramzy, 2007; Molyneux, 2009). In addition, it can take years for levels of CFA to rise to detectable levels (Lammie ***et al.***, 1998). In high prevalence settings children as young as 2 years old have tested as CFA positive (Lammie ***et al.***, 1998). However it is unknown how long it would take in a low prevalence setting.

2.4.2.1 NOW® filariasis immunochromatographic test (ICT)

During the monitoring stages of LF elimination the introduction of the field test, the NOW® Filariasis ICT, was a key milestone for LF diagnostic assays (Weil ***et al.***, 1997). It allowed quick and easy diagnosis in the field, provided that the manufacturer's instructions were clearly followed (Rajgor ***et al.***, 2002). The ICT is based on capillary migration of approximately 100 µL of whole blood. The blood migrates down the card, and any ***W. bancrofti*** antigen present in the blood sample will bind to the polyclonal or monoclonal

antibodies impregnated in the filter of the card producing a visible reaction (Weil ***et al.***, 1997). The efficacy of the ICT has been reviewed by many researchers reporting sensitivities up to 100% and specificities of 96.3% in high prevalence settings (Nguyen ***et al.***, 1999; Phantana ***et al.***, 1999; Simonsen and Dunyo, 1999).

It needs to be stressed that reported high sensitivity and specificity of the ICT was always in high prevalence settings. Now that countries are nearing elimination the ICT has inherent problems due to a lack of sensitivity in low prevalence settings (Section 2.4.2).

2.4.2.2 Og4C3 assay

The Og4C3 ELISA is based on a monoclonal antibody and is a commercially available CFA assay (Tropbio, Australia). Although a laboratory is needed for the ELISA, filter papers can be used for sample collection as the assay is adaptable for whole blood elutions (Hoti ***et al.***, 2002). This makes the assay useful for field conditions, where it is often difficult to collect and store serum samples. Although reported sensitivity has varied, it has been reported to reach 100% in high prevalence settings (Lammie ***et al.***, 1994). However, the decline in LF prevalence compromises the sensitivity of the Og4C3 ELISA.

2.4.3 Microfilarial (Mf) Assays

Mf assays are important to assess whether an individual is a potential source of ongoing LF transmission. Persons who are amicrofilaraemic (30-50% of LF cases), but do harbour adult worms, need to be treated for their own health, but do not pose a threat to others. Amicrofilaraemic infections occur because of two reasons:

1) The individuals is infected with single sex worms, thus no mating can occur and Mf are not produced (Njenga ***et al.***, 2007a); or,
2) The female worms are immature and are incapable of reproduction (Njenga ***et al.***, 2007a).

Mf assays currently available include the thick blood smear, Knott's concentration method, membrane filtration techniques, and, more recently, PCR techniques. The advantages for current Mf assays, especially the thick blood smear, include the high specificity (100%), the low cost and the requirement for little infrastructure (Weil and Ramzy, 2007). However, one significant disadvantage, as prevalence reduces, is the requirement for larger sampling sizes due to the inherent low sensitivity of the assay (Weil and Ramzy, 2007). This would make sampling laborious, especially since trained laboratory technicians are required (reviewed in Mendoza ***et al.***, (2009)). Concentrating Mf, using the Knott's concentration method, may improve sensitivity, but unlikely to the levels required for surveillance and, more importantly, venous blood samples are required.

Membrane filtration assays concentrate Mf resulting in a sensitivity of one Mf/mL of blood. The blood, diluted in water to lyse the red cells, is passed through a cellulose filter that traps Mf, making the assay more sensitive than the Knott's concentration method (Bell, 1967; Chularerk and Desowitz, 1970; Desowitz and Southgate, 1973; Desowitz ***et al.***, 1973; Abaru and Denham, 1976; Shibuya ***et al.***, 1977). The advantage of this technique is that Mf are still viable. Therefore, upon separation and concentration, Mf can be used for further study, or stained and viewed for counting. However, there are disadvantages to this technique. The blood must be immediately processed upon collection, filters are expensive and, most importantly, venous blood is required (Melrose, 2004). Venous blood collection increases the risk of needlestick injury, which is of importance in areas where there is potential transmission of blood-borne infectious diseases such as HIV, Hepatitis B and C (Rodriguez-Perez ***et al.***, 1999).

Mf assays are relatively insensitive, compared to other diagnostic tools, but sensitivity can be increased when using PCR techniques albeit not significantly (Cox-Singh ***et al.***, 2000; Hassan ***et al.***, 2005). The PCR amplifies the Mf DNA (Cox-Singh ***et al.***, 2000; Hassan ***et al.***, 2005). Whichever Mf assay is chosen, the disadvantage remains concerning sample collection times, sampling sizes and the relative insensitivity compared with CFA and anti-filarial antibody assays. Thus, exclusive reliance on this technique will result in underestimating prevalence of infection and, if used as a future surveillance tool, possibly exclusion of areas that require treatment.

2.4.4 Anti-filarial Antibody Assays

2.4.4.1 Introduction

Detection of antibodies as a diagnostic tool is beneficial for individuals residing in non-endemic areas, since a positive test would be indicative of exposure and the need for treatment. In the past, anti-filarial antibody assays in endemic areas proved less useful, since the community was constantly exposed to infective mosquitoes and thus expected to be anti-filarial antibody positive (Njenga ***et al.***, 2007a). However, with the approach of the end of the MDA, the age prevalence should have shifted as LF transmission is interrupted. Consequently, younger individuals born following implementation of MDAs should have lower exposure to the parasite, than those in high prevalence settings, and therefore prevalence should be either low or non-existent in these individuals (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). This now makes the anti-filarial antibody assay a potential tool for monitoring LF exposure for both endemic and non-endemic countries.

Anti-filarial antibody assays were developed as early as 1980 and a wide range of crude filarial parasite antigens were utilised (reviewed in Melrose, (2004)). The problem observed with these assays was cross-reactivity with other filaroid species, including ***L. loa*** and ***Onchocerca*** sp. and other helminths such as ***Strongyloides*** sp. (Langy ***et al.***, 1998). This was a

problem since in LF endemic areas poly-parasitism is common (Utzinger and de Savigny, 2006). Since then, recombinant antigen technology has been utilised with promising results (Lammie ***et al.***, 2004).

2.4.4.2 Recombinant antigen assays

The addition of recombinant antigen detection systems into the repertoire of potential LF diagnostic tools has been a turning point for the LF programme. The sensitivity and specificity are far superior to the antibody assays reliant on crude antigen (Lammie ***et al.***, 2004). The three main recombinant antigens utilised are SXP-1 (both ***B. malayi*** (Bm) and ***W. bancrofti*** (Wb) orthologues), ***B. malayi*** recombinant antigen 1 (BmR1), and Bm14 (Dissanayake ***et al.***, 1992; Chandrashekar ***et al.***, 1994; Wang ***et al.***, 1997; Rahmah ***et al.***, 1998; Rao ***et al.***, 2000; Rahmah ***et al.***, 2001a; Lalitha ***et al.***, 2002; Rahmah ***et al.***, 2007). The latter is the recombinant antigen utilised in the Filariasis CELISA. Recently, trials in India have been conducted to ascertain the usefulness of a recombinant antigen sourced from the bovine filarial parasite ***Setaria cervi*** (Singh ***et al.***, 2010). The authors concluded that currently available commercial kits, such as the Filariasis CELISA, are not feasible for the Indian LF programme because of issues with cost and availability, yet the authors do not indicate how they came to this conclusion. Secondly, a major shortcoming from their study was that only sera from infected individuals were used to assess sensitivity and specificity of the assay without consideration of endemic uninfected individuals. Thirdly, the antigen was shown to cross react both with ***Ascaris*** sp. and hookworm when

detecting IgG_1 (Singh ***et al.***, 2010), making it less useful in areas co-endemic for these parasites (Utzinger and de Savigny, 2006).

A comparison between the antigens is tabulated (Table 2.1). The overall findings from the comparison highlight that when countries enter surveillance mode, and thus low prevalence settings, antigen assays based on the recombinant antigen Bm14 would be most appropriate. Assays based on BmR1 were insensitive for detecting Bancroftian filariasis, as expected, and thus are not suitable in these endemic areas (Lammie ***et al.***, 2004). In a multicentre trial it was established that whilst SXP-1 had comparable sensitivity to Bm14 for detection of Mf positives, 20% of assays based on the former recombinant antigen resulted in a "no consensus" invalid result (Lammie ***et al.***, 2004). This is not appropriate for surveillance. Thirdly, and most importantly, SXP-1 recombinant antigen has reported low sensitivities for amicrofilaraemic CFA positive individuals (Dissanayake ***et al.***, 1992). This would be detrimental in low prevalence settings when the proportion of Mf positive individuals is reduced. Detection systems based on Bm14 are more appropriate for detecting late exposure or early pre-patent infection (Ramzy ***et al.***, 1995; Weil ***et al.***, 1999). Consequently, Bm14 is the most appropriate recombinant antigen as an early warning detection system both for identifying residual endemnicity and during surveillance mode.

Table 2.1: Recombinant antigen based antibody assays. The three main recombinant antigens available are SXP-1 (both ***B. malayi*** and ***W. bancrofti*** orthologues), BmR1 and Bm14.

Recombinant antigen	**Commercially available**	**Antibody detected**	**Reported sensitivity for Bancroftian filariasis**	**Reported sensitivity for Brugian filariasis**	**Cross reactivity with other helminths or filarial parasites**	**Disadvantages**	**References**
SXP-1 (Bm or Wb) **Note:** Truncated version SXP-1 called Wb14	1) WbSXP SXP Cassette (Span Diagnostics, Ltd)	IgG_4	1) WbSXP 85 – 92% with Mf positive sera	1) WbSXP 39 - 91% with Mf positive sera	**The SXP-1 antigen** 0-2% with other helminth infections 40 – 60% with other filarial parasites	Shown to have low sensitivity for amicrofilaraemic CFA positive individuals. In the cassette format there is potential for "no consensus" invalid result about 20% of the time. Increased sensitivity using ELISAs with two coated wells increases cost. PanLF (BmSXP) has low sensitivity for Bancroftian filariasis	Dissanayake ***et al.***, 1992; Theodore ***et al.***, 1993; Dissanayake ***et al.***, 1994; Rao ***et al.***, 2000; Basker ***et al***., 2004; Lammie ***et al.***, 2004; Abdul Rahman ***et al.***, 2007; Rahmah ***et al.***, 2007; Pandiaraja ***et al.***, 2010
	2) BmSXP PanLF Cassette		2) BmSXP 12% with Mf positive sera	2) BmSXP 94% with Mf positive sera			

Table 2.1 *Continued*

Recombinant antigen	Commercially available	Antibody detected	Reported sensitivity for Bancroftian filariasis	Reported sensitivity for Brugian filariasis	Cross reactivity with other helminths or filarial parasites	Disadvantages	References
BmR1	Brugia Rapid (Malaysian Bio-Diagnostics Research)	IgG_4	12-57% with Mf positive sera	> 90% with Mf positive sera	0% with other helminth infections and filarial parasites	Not useful in Bancroftian endemic areas such as the Pacific.	Rahmah ***et al.***, 2001a; Rahmah ***et al.***, 2001b; Rahmah ***et al.***, 2003a; Rahmah ***et al.***, 2003b; Lammie ***et al.***, 2004; Rahmah ***et al.***, 2004
Bm14	Filariasis CELISA (Cellabs Pty Ltd)	IgG_4	85 – 90% with Mf positive sera	96% with Mf positive sera	0% with other helminth infections 69-78% with other filarial parasites	Commercial assay needs to be field friendly. Commercial assay needs to be investigated for its potential use in endemic areas.	Chandrashekar ***et al.***, 1994; Ramzy ***et al.***, 1995; Weil ***et al.***, 1999; Lammie ***et al.***, 2004; Weil ***et al.***, 2010

2.5 NEW LABORATORY TEST: FILARIASIS CELISA

2.5.1 Introduction

The Filariasis CELISA assay is an IgG_4-specific ELISA in which the plates are coated with the recombinant antigen Bm14 (Cellabs Pty Ltd, Brookvale, NSW, Australia). The commercially available assay was adapted from the research-based Bm14 assay prototype (Chandrashekar ***et al.***, 1994) and has been demonstrated to be highly sensitive and specific using serum (Weil ***et al.***, 2010).

The Bm14 gene belongs to a family of genes encoding proteins that are strong immunogens (Rao ***et al.***, 2000) and was originally isolated from a cDNA library in 1992 for its potential application in LF diagnostic tools (Dissanayake ***et al.***, 1992). These initial studies demonstrated the affinity of antibodies isolated from microfilaraemic individuals for the expressed 132 kDa recombinant antigen (Dissanayake ***et al.***, 1992; Chandrashekar ***et al.***, 1994). Use of Bm14 in the research-based assay prototype has shown Bm14 to be reactive with sera from patients with Brugian or Bancroftian filariasis with reported sensitivities of 96% and 91% respectively (Lammie ***et al.***, 2004). No reaction was reported with 19 serum samples from ***Strongyloides*** patients (Lammie ***et al.***, 2004). Unfortunately, Bm14 cross-reacted with 72% of the ***L. loa*** and ***O. volvulus*** positive sera limiting its usefulness in co-endemic African regions (Lammie ***et al.***, 2004).

2.5.2 Cross-Reactivity and Inter-Laboratory Variation

Although the Filariasis CELISA anti-filarial antibody assay has been commercially available as a diagnostic tool since 2006, application to large population sizes in field studies has not been thoroughly assessed. Currently, all previous published research utilising the recombinant antigen Bm14 in field studies has been with the research-based assay prototype (Li ***et al.***, 2004; Washington ***et al.***, 2004; Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Njenga ***et al.***, 2007a; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). In these studies there are two main disadvantages by employing the research-based Bm14 assay prototype. No direct intra- or interlaboratory comparisons can be concluded since standardised reagents nor stringent quality control (QC) practices were enforced, such as those seen with commercially available reagent kits (Weil ***et al.***, 2010). Secondly, the commercial assay has been shown to have higher sensitivity than the research-based Bm14 assay prototype since a different substrate detection system is utilised (Weil ***et al.***, 2010).

Issues with poly-parasitism in LF endemic areas will always affect the potential specificity of an antibody assay (Utzinger and de Savigny, 2006). Therefore, each new assay requires cross-reaction studies to eliminate the possibility of this potential problem. Recently, a multi-centre trial addressed this issue and concluded that not only did the Filariasis CELISA achieve high specificity, but there was little inter-laboratory variation (Weil ***et al.***, 2010). I was fortunate enough to be a member of the research team (Chapter 4) (Weil

et al., 2010). The favourable conclusions from the study highlighted the opportunity to explore the Filariasis CELISA for its potential to be included in future surveillance strategies.

2.6 POST-MDA: DETERMINING IF COUNTRIES ARE FILARIASIS FREE

2.6.1 Introduction

Determining accurately if countries are LF-free is crucial for the success of the LF programme. Consequently, thresholds are required to define a point where the acquisition of new adult worms is lower than the death rate of existing worms (Stolk ***et al.***, 2006). This point represents a threshold where the low density of Mf is insufficient for ongoing LF cycling (WHO, 2007b). There is strong evidence that the current thresholds for LF elimination, defined by WHO, are outdated and too high to represent accurate LF elimination (Gambhir ***et al.***, 2010) (Section 2.6.2).

Recent mathematical modelling, based on ***Culex*** sp. transmission, hypothesised that a more stringent threshold of < 0.5% Mf prevalence may be required to interrupt transmission (Xu ***et al.***, 1997; Michael ***et al.***, 2006a; Stolk ***et al.***, 2006), especially because of the threat of resurgence (Section 2.6.2) (Chanteau ***et al.***, 1995; Weil ***et al.***, 1999; PacELF, 2006; Plichart ***et al.***, 2006). On the contrary, areas of India where < 0.5% Mf prevalence was reached showed persistent Mf in the population for 20 years

(Ramaiah ***et al.***, 2009). However, this was hypothesised to be due to scattered areas where Mf prevalence ≥ 1% (Ramaiah ***et al.***, 2009). In Haiti, achieving Mf prevalence < 1% after seven rounds of MDA was not associated with interrupted transmission, since subsequent studies in children revealed a CFA prevalence exceeding 10% (Boyd ***et al.***, 2010). It was speculated that the continuing transmission was due to the high number of systematic non-compliers (Boyd ***et al.***, 2010). Additionally, studies have demonstrated that, despite low Mf prevalence, transmission is ongoing because of the high density of vectors (Das and Vanamail, 2008). This reiterates the necessity for differing thresholds in different epidemiological settings.

Complete absence of filarial parasites is not required to make the decision to stop MDAs, but rather reduction to a threshold at which transmission is interrupted (Michael ***et al.***, 2004; Duerr ***et al.***, 2005; Gambhir and Michael, 2008). Two different thresholds were set by GPELF and PacELF as initial targets for countries to reach and these were < 1% Mf prevalence or < 1% CFA prevalence of the population respectively (WHO, 2005; PacELF, 2006). Once achieved, both GPELF and PacELF defined an interruption in transmission permanently as < 0.1% CFA prevalence in children (WHO, 2000; PacELF, 2006). The age group targeted in the Pacific was first level school entrants, approximately 5 years old (Huppatz, 2008). The age group outlined by GPELF was those born after the initiation of effective MDAs, usually those born after implementation of round four, approximately 2 to 4 year olds (WHO, 2005). To assess the efficacy of each MDA round,

Pacific countries were required to implement surveys. The initial survey, the A survey, was designed to provide a baseline of LF endemnicity in each country based on the WHO recommendation of implementation units (IU) (WHO, 2005; PacELF, 2006). The IU defined a geographical area designated by a level of administrative unit, such as health districts. If LF is found to be endemic in the IU, then the decision is made to implement MDA countrywide (WHO, 2005). Except for PNG, IU in each country consisted of the entire country (PacELF, 2006). Sentinel sites were chosen, as per the WHO protocol, and a midterm survey was implemented (B survey) (PacELF, 2006).

The midterm survey, using CFA prevalence in chosen sentinel sites, was to monitor the progress of MDAs and make any necessary adjustments. After the fifth round of MDAs a C survey was implemented. The C survey was a countrywide prevalence survey, testing randomly selected clusters, to assess if the prevalence of CFA had fallen below the required 1% (PacELF, 2006). If not, further MDA rounds would be planned. In addition to prevalence surveys, disease burden was assessed and those already symptomatic were clinically managed (WHO, 2005).

If Pacific countries reached < 1% of CFA prevalence, transmission interruption was assessed using lot quality assurance sampling (LQAS) of children (WHO, 2005). LQAS was originally based on the concept of the production line industry, whereby the population is divided into IUs and individuals are tested until a positive is found (Pani ***et al.***, 2000; WHO, 2000).

Once a positive is found the IU is designated as endemic (Pani ***et al.***, 2000). The goal of the LQAS was to conduct a large community ICT survey of 3000 first year school children. The arbitrary threshold definition for interruption of transmission was originally set at < 0.1% CFA prevalence of children aged between 5 and 6 years old (WHO, 2005; PacELF, 2006). However, as countries reach the endpoint of their MDA campaigns, and prevalence declines, intense debate surrounds the methodology to demonstrate interruption.

A number of Pacific countries have completed five rounds of MDA and their prevalence of LF is below, or close to, the < 1% antigenaemia target set by PacELF (Huppatz ***et al.***, 2009). These countries now move into the next phase of the programme that entails:

- Detection and elimination of foci of residual endemnicity (referred to as "hot spots"); and,
- Setting up surveillance to provide an early warning system of renewed transmission (resurgence).

The phase of determining whether an area is LF-free, and can enter post-MDA surveillance, (WHO, 2009) poses particular challenges: "hot spots" may be scattered and ill-defined, prevalence of infection may be low, and the diagnostic tools that were so successful in the earlier phase of the programme may no longer be suitable because of issues such as low sensitivity (Chanteau ***et al.***, 1994; Rocha ***et al.***, 1996; Grady ***et al.***, 2007; Njenga ***et al.***, 2007a; Molyneux, 2009). It has also been strongly suggested

that the current WHO guidelines necessitating continuing MDA rounds if one in 3000 children born after the MDA is positive for CFA, via LQAS, (WHO, 2005) is excessive and beyond what is needed for successful LF elimination (Weil and Ramzy, 2007). Secondly, there is the potential for ICT positives to persist for years if the initial worm burden was high or if the DEC therapy was too low (Weil ***et al.***, 1988; Weil and Ramzy, 2007). The current guidelines do stipulate that adaptation may be required as future research becomes available (WHO, 2005).

Following the decision to cease MDAs, countries are required to carry out surveillance for at least five years (WHO, 2005). Surveillance of endemic countries needs to be continuous which can be difficult without adequate resources and funding. Secondly, continuous sampling with no benefits to participants raises ethical issues. To overcome these issues, it has been suggested that surveillance sampling should be conducted using mosquitoes (Laney ***et al.***, 2010) or children of primary school age (Huppatz, 2008). However, there is still a lack of data linking these infective mosquitoes to an infected population (Mladonicky ***et al.***, 2009). Consequently, the ideal sampling population still stands as endemic individuals. To address the ethical issue of testing the whole population, surveys can target school children around the ages of 4 to 6 years, born after the last round of MDA. These children should be anti-filarial antibody negative if transmission has been interrupted successfully (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009).

At the 2007 PacELF meeting a five year plan was drafted (WHO, 2007a). Countries on the brink of elimination entered monitoring and surveillance mode until 2012 based on the proposal by PacCARE, whereas other countries with ≥ 1% CFA prevalence of population planned further MDAs (WHO, 2007a). The elimination strategy for PNG was lengthened until 2020 (WHO, 2007a). Surveillance mode was planned for a minimum of four years, every second year, in countries according to the LF surveillance strategy for the PICT (Huppatz, 2008).

The assays that have been suggested as a monitoring tool in this phase of the programme have been antibody serology, namely with the Filariasis CELISA assay (Joseph and Melrose, 2010), PCR of mosquitoes and mosquito dissection tools (Laney ***et al***., 2010), or CFA testing in children (Huppatz, 2008). Currently, only standardised Mf and CFA testing are recognised by the WHO as diagnostic assay choices for the elimination programme until other diagnostic tests are assessed for their suitability (WHO, 2005). Consequently, to assess interruption of transmission, CFA testing in children was part of the LF surveillance strategy for the PICT recently outlined for PacCARE dubbed “Child Transmission Surveys” (CTS) (Huppatz, 2008). It entails ICT testing of children aged between 5 and 6 years in either community based or school based surveys. Confirmed positives are followed up by testing surrounding households from the positive child’s household of residence, either 24 houses or a radius of 200 m, to identify the potential Mf positive case, termed “close contact testing” (Huppatz, 2008). The logistics of CTS as a means for identifying residual

endemnicity and the potential to use this strategy for ongoing surveillance are investigated as part of the thesis.

Additionally, presence of the highly efficient day-biting ***Ae. polynesiensis*** also makes LF elimination in the Pacific challenging (Burkot ***et al.***, 2002). This is because of the characteristic of limitation (Section 2.1) and the lack of spatial clustering of cases observed for this day-biting vector, concluded to be due to movement of people during the day (Mladonicky ***et al.***, 2009). Consequently, the current LF surveillance strategy for the PICT and close contact testing may not be feasible in these endemic areas.

There are other notable problems with the proposed surveillance strategy including the choice of diagnostic tool. There are concerns about the limits of sensitivity of CFA testing (Section 2.4.1), which will negatively impact upon the programme's goal of sustainable LF elimination. Secondly, stringent surveillance is warranted for the Pacific because of evidence of resurgence in the past (Section 2.6.2). Overall, a successful surveillance strategy requires a sensitive diagnostic tool which will also allow LF programme managers to trace the source of the outbreak. It is the aim of this research to ascertain the usefulness of measuring exposure in children.

In summary, highlighted gaps in knowledge in this stage of the programme include:

1) Whether LF elimination can be sustainable over time;

2) If a < 1% antigenaemia target or < 1% Mf prevalence will completely interrupt cycling between host and mosquito;
3) Which diagnostic tools would be appropriate to monitor these concerns; and,
4) Whether current surveying methods are appropriate during surveillance.

Finding the answers to these questions has become an urgent priority for GPELF and PacELF as they will determine the success of elimination.

2.6.2 Resurgence of Lymphatic Filariasis Following Elimination: an Historical Perspective

Resurgence is a threat to the success of global LF elimination. It should be of concern to PacELF that of those four countries where resurgence has been recorded, three were PICT; French Polynesia, the Cook Islands, and Samoa (Kimura ***et al.***, 1985; PacELF, 2006; Plichart ***et al.***, 2006). It is no coincidence that these Pacific countries all have ***Ae. polynesiensis*** as the endemic vector, which has been identified as a key impediment to successful LF elimination (Kyelem ***et al.***, 2008). Consequently, why is vector control not an additive measure in these endemic areas since vector control has been identified as necessary for elimination (Bockarie ***et al.***, 2009a)? Due to the day-biting tendencies of ***Ae. polynesiensis***, vector-control strategies were thought to be challenging since traditional interventions used in areas of nocturnal periodicity, such as bednets (Burkot ***et al.***, 2002) and control of

breeding sites around the household, were thought not to be effective (Bockarie ***et al.***, 2009a). Secondly, the vector preferentially remains outdoors reducing the efficacy of indoor insecticide spraying (Ramalingam, 1968; Samarawickrema ***et al.***, 1987a; Bockarie ***et al.***, 2009b). Recently, the potential for source reduction campaigns in American Samoa has been explored, where ***Ae. polynesiensis*** is endemic (Lambdin ***et al.***, 2009). This strategy should be implemented in other ***Ae. polynesiensis*** endemic regions if resurgence is to be avoided.

French Polynesia, the Cook Islands, and Samoa all achieved < 1%
Mf prevalence of the population prior to the establishment of GPELF (WHO/SPC, 1974; Ichimori, 2001; Esterre ***et al.***, 2005). Unfortunately, by the 1990s during baseline prevalence surveys, as required when PacELF was established, re-establishment of LF transmission was revealed (PacELF, 2006).

The evidence of resurgence in the Pacific reiterates:

1) The necessity for different thresholds for defining cessation of LF transmission in different epidemiological settings;
2) The necessity for evaluating the inclusion of vector control strategies for every country in the Pacific; and,
3) The necessity for effective surveillance strategies and accompanying diagnostic tools to prevent resurgence or, at the very most, quickly control any identified outbreaks.

I propose that the latter would be solved by monitoring anti-filarial antibody levels in children.

2.6.3 PCR and Mosquito Dissection Tools for Monitoring Lymphatic Filariasis Transmission

There are two methods for detecting LF in vector populations: dissection and PCR (Goodman ***et al.***, 2003). Both methods rely on either the detection of Mf following a blood-meal, which may not be a true representation of ongoing transmission, or the detection of iL3 (reviewed in Pedersen ***et al.***, (2009)).

Mosquito dissection is the gold standard for measuring LF in the vector population (Goodman ***et al.***, 2003). Unfortunately, this technique relies on a trained entomologist, is costly and laborious, and less sensitive than newer PCR techniques (Manguin ***et al.***, 2010). The PCR techniques, referred to as molecular xenomonitoring, are based on pooling mosquitoes to detect filarial parasites (Goodman ***et al.***, 2003; Pedersen ***et al.***, 2009; Manguin ***et al.***, 2010). While it is considered to be more sensitive than older techniques, mosquito trapping is required and this can be a laborious task (Goodman ***et al.***, 2003). Molecular xenomonitoring is not a direct measure of transmission, since ingested Mf are detected, not iL3 (Pedersen ***et al.***, 2009). Not all ingested Mf develop into iL3, the latter is the stage that infects the human host (Southgate and Bryan, 1992). Subsequent advances in technology have allowed the specific amplification of iL3 ribonucleic acid (RNA) using reverse transcriptase PCR (RT-PCR) (Laney ***et al.***, 2010). For the first time,

researchers will be able to quantify the infectivity ratio of mosquitoes. However, in order to provide useful information for programmatic decisions, the infectivity of mosquitoes needs to be related to the transmission dynamics of LF in the community.

During this end-stage of LF elimination it is not only important to have a surveillance tool that detects any resurgence or hotspots, but also to explain epidemiologically the relationship between resurgence and geographical areas requiring another round of treatment. For example, if the resurgence is identified using vector methods, then how does the "infective mosquito" relate to which individuals are potentially exposed and infected in a geographical area? These studies are necessary to detect and manage the infected individuals requiring treatment, before LF transmission spreads and intensifies. One such study investigated the geographical relationship between LF transmitting mosquitoes using molecular xenomonitoring and Mf/CFA positive individuals, but found no spatial relationship (Mladonicky ***et al.***, 2009). This lack of geographical relationship between infective mosquitoes and infected individuals poses a potential problem for using mosquito techniques as a surveillance tool. To date, the relationship between detecting iL3, using RT-PCR, and LF transmission has not been determined.

There are other disadvantages to using mosquito monitoring as a surveillance tool. Both dissection and PCR techniques are expensive, especially when the numbers of mosquitoes required increases as the

prevalence declines (Pedersen ***et al.***, 2009). It has already been demonstrated that dissection techniques are not suitable in low prevalence settings, making this method inadequate as a potential surveillance tool (Goodman ***et al.***, 2003). Secondly, the necessary equipment and expertise is not usually available in these under-resourced developing countries (Pedersen ***et al.***, 2009). Thirdly, thresholds to define interruption of LF transmission via sampling the mosquito population need to be investigated; differing for each vector endemic area.

2.6.4 Choosing the Right Diagnostic Tools for Surveillance

The gold standards in the MDA phase of the LF elimination programme, that is the Mf and CFA tests, are not as useful for identifying residual endemnicity due to the slow-evolving nature of disease (Durrheim ***et al.***, 2003; Melrose ***et al.***, 2004; Washington ***et al.***, 2004). There may be many months of exposure to infective mosquitoes before infection occurs and that person becomes CFA positive, and another lag phase before that person becomes Mf positive, which means a more timely “early warning system” for continuing or renewed infection is required (Ramzy, 2002; Durrheim ***et al.***, 2003; Rawlins ***et al.***, 2004; Grady ***et al.***, 2007; Weil and Ramzy, 2007). This is especially true in those areas where ***Ae. polynesiensis*** is the vector, since it is highly efficient in LF transmission (Ichimori and Crump, 2005) and is the vector present in certain areas where previous resurgence has been demonstrated (Plichart and Legrand, 2005).

To investigate the best diagnostic tool to use for the surveillance phase, known hotspots of residual endemnicity should be studied. This is to provide an accurate picture of how the diagnostic tools complement each other in known prevalence settings. These areas could be identified by the LF programme managers for each country, as they are familiar with problem areas of low MDA coverage or non-compliance. By combining currently available diagnostic tools testing antigenaemia, microfilaraemia, and antibodies coupled with global positioning system (GPS) mapping, a spatial relationship may be demonstrated (Section 2.7). From this data, an informed decision can be made as to the usefulness of measuring levels of exposure in children (anti-filarial antibody assays) as a means for identifying the extent of possible resurgence. This possible spatial relationship will differ for each endemic area depending on the vector present. To be an acceptable diagnostic test for inclusion into the LF programme, antibodies need to be measured with a standardised commercial kit, such as the Filariasis CELISA.

2.6.4.1 Filariasis CELISA anti-filarial antibody assay as the answer

The Filariasis CELISA has been commercially available since 2006 (Section 2.5). The commercial assay was based on the research Bm14 assay prototype, which was extensively used in the past in field settings with promising results (Li ***et al.***, 2004; Washington ***et al.***, 2004; Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). The Filariasis CELISA is yet to be used in field studies to assess its efficacy in high or low prevalence.

Previously, the assay prototype was concluded to be applicable for large sampling sizes for monitoring LF transmission in areas of high prevalence (Grady ***et al.***, 2007; Tisch ***et al.***, 2008). An independent study demonstrated that anti-filarial antibody levels in a population decreased with age and could serve as a good indicator of decreasing LF transmission (Weil ***et al.***, 1999). Now that the assay prototype has been adapted for commercial use, further investigation is required to ensure the applicability of the Filariasis CELISA as a potential LF diagnostic tool in the low prevalence phase. Initial laboratory studies, using serum, have shown promise (Weil ***et al.***, 2010). However, for the feasible inclusion into the LF programme the assay needs to be more field-friendly. This could be achieved by using whole blood dried onto filter paper, which has been accomplished with other ELISAs (Santhanam ***et al.***, 1989; Terhell ***et al.***, 1996; Itoh ***et al.***, 1998).

Whether the surveillance phase monitors Mf, CFA, and/or anti-filarial antibody prevalence, LF programme managers for each country need to have enough information in order to target LF treatment effectively and ethically. If they treat too small an area, they run the risk of missing possible cases that have been exposed, but are still within the pre-patent period of infection (CFA negative). If they treat too large an area, any long term surveillance programme may not be economically feasible. The economic feasibility is a huge factor, since previous studies have already shown LF is capable of resurging to pre-programme levels when monitoring is not used (Harb ***et al.***, 1993; Chanteau ***et al.***, 1995; Weil ***et al.***, 1999). Therefore, studies need to be carried out in a known area of residual endemnicity of low

prevalence. These studies need to incorporate GPS mapping, CFA testing, Mf testing, and the Filariasis CELISA anti-filarial antibody assay, thus allowing an informed decision about the most appropriate diagnostic tool for future surveillance. An anti-filarial antibody assay as a future surveillance tool would only be useful if there was a relationship between exposed children (clusters of cases) and an index case. This would allow the LF programme managers to effectively target LF treatment to specific geographical areas.

2.7 USE OF GPS MAPPING WITH DIAGNOSTIC TOOLS TO ASSESS THE EXTENT OF LYMPHATIC FILARIASIS TRANSMISSION

As programmes reach their elimination stage, detailed prevalence mapping is crucial to allow planning for the surveillance stage (Baker ***et al.***, 2010). This is to visualise areas that require targeted intervention. There is the potential for the maps to include geo-statistics and climate-based risk models to guide control efforts (Brooker, 2007; Simoonga ***et al.***, 2009). GPS mapping and spatial analysis has been successfully used in other infectious diseases, including leprosy, and other mosquito-transmitted diseases such as malaria and dengue (Nuckols ***et al.***, 2004; Sithiprasasna ***et al.***, 2004; Fleming ***et al.***, 2005; Gaudart ***et al.***, 2006; De Souza Dias ***et al.***, 2007). Using these already established techniques in currently known low LF prevalence areas, information can be gathered as to whether there is a spatial relationship between a Mf positive case and the outlying people being exposed (anti-filarial antibody positive children). To date, only one study conducted in an

Ae. polynesiensis endemic area has been published and this showed no spatial relationship between CFA positive individuals residing in households (Mladonicky ***et al.***, 2009). In areas where the vectors are night-biters spatial relationships between households where infected individuals reside have long been established (Walter, 1974; Gad ***et al.***, 1994; Das ***et al.***, 1997; Alexander ***et al.***, 1998; Weil ***et al.***, 1999).

The link between infected and exposed individuals has been explored in one study to date in a ***Culex quinquefasciatus*** endemic area (Washington ***et al.***, 2004). Washington ***et al***. (2004) demonstrated that an increase in distance from a household with CFA positive individuals was inversely proportional to anti-filarial IgG_1 levels, although this measurement was for all individuals not just children. A limitation of the study was the format of the ELISA, based on detecting IgG_1 using crude antigen. Both the isotype and the crude antigen are prone to cross reactions (Sections 2.3.3.2.1 and 2.4.4.1). Thus, the absence of adequate information regarding the link between antibody serology and infected individuals deems further investigation.

2.8 SUMMARY

In summary, key gaps in knowledge have been highlighted that need addressing. These include, but are not limited to:

1) What is a feasible surveying method for future surveillance strategies and/or identifying areas of residual endemnicity?

2) What is an appropriate sensitive diagnostic method allowing early warning of resurgence or to identify areas of residual endemnicity?
3) Will this diagnostic test allow easy detection of the extent of resurgence or residual endemic foci?
4) Is the historical evidence of resurgence due to consistent non-compliance? Who are these non-compliers?

If the Filariasis CELISA anti-filarial antibody assay can be applied in the field, demonstrated to accurately detect anti-filarial antibody positive individuals in low prevalence settings, and link early exposure geographically to index cases then findings from this research will be crucial for countries facing the end point of their LF programme; not only to identify areas where residual endemnicity is occurring, but also as a tool for future surveillance. The GPS spatial analysis should give an indication of the radius from the exposed anti-filarial antibody positive child to the infective (Mf positive) individual, allowing targeted drug therapy to individuals within this geographical area. This allows swift action before a resurgence takes hold and an outbreak occurs. Earlier anti-filarial antibody studies were inaccurate when based on crude antigen. Consequently, the newer Filariasis CELISA anti-filarial antibody assay based on recombinant antigen should be more specific, giving a more precise representation of the sero-epidemiology of disease. It is imperative at this stage in the global elimination programme that the correct diagnostic tool is used to ensure cost-effective protection for future generations against this debilitating disease.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 STUDY SITES

Study sites for the research were spread across the South Pacific (Figure 3.1) encompassing Vanuatu, Tonga, Tuvalu, Samoa and PNG where LF is endemic (PacELF, 2006).

Figure 3.1: Map of the South Pacific. Study sites for the research included Vanuatu, Tonga, Tuvalu, Samoa and PNG (Bureau of Meterology, 2007).

3.2 LABORATORY DIAGNOSIS OF LYMPHATIC FILARIASIS

3.2.1 Reagents and Consumables

Generic reagents and laboratory consumables used were readily available from most scientific distributors. Main commercial suppliers included Sigma-Aldrich (Castle Hill, New South Wales (NSW), Australia), Bio-Rad Laboratories (Gladesville, NSW, Australia) and BD (Becton, Dickinson and Company, North Ryde, NSW, Australia). Other common consumables such as cotton balls and bandaids were bought from a local pharmacy. Transport ziplock bags were sourced from the local supermarket. The three diagnostic assays used for the research were NOW® filariasis ICT, (Binax, USA), the Og4C3 ELISA (Tropbio, Townsville, Australia) and the Filariasis CELISA (Cellabs, Brookvale, Australia).

3.2.2 Blood Collection and Storage

For all laboratory methods only a fingerprick sample of blood was required. The finger chosen was initially cleaned with an ethanol swab and pricked using a lancet. A 100 µL calibrated capillary, provided with the ICT kits, was used to collect the first flow of blood. The rest of the blood was collected onto a Tropbio filter paper disc (Figure 3.2; Tropbio Pty Ltd, QLD, Australia). All six protrusions were saturated with blood, to give a volume of 10 µL of blood on each protrusion, thus a total of 60 µL. When Mf status was also

investigated, a three line blood film was prepared (Section 3.2.4) (Sasa ***et al.***, 1976).

The filter papers were dried overnight (Figure 3.2) and placed into separate ziplock bags, bundled together into a larger ziplock bag with desiccated silica gels sachets. This was to prevent moisture affecting sample integrity. Filter papers were transported back to Australia at ambient temperature (22 - 25^{o}C) (Coltorti ***et al.***, 1988) and stored at -20^{o}C upon arrival. Following preparation of glass slides for Mf detection, slides were left to dry overnight, wrapped, and transported at ambient temperature to James Cook University (JCU), Australia.

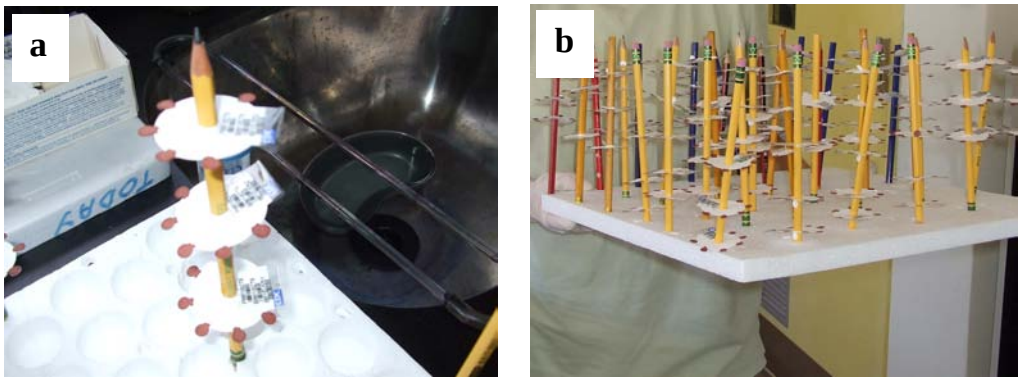


Figure 3.2: Preparation and drying of filter paper samples. (a) The six protrusions of each filter paper were thoroughly soaked to give 10 µL of blood and threaded onto a pencil for drying. (b) The arrangement of pencils for drying of filter papers.

3.2.3 Elution of Filter Paper

3.2.3.1 Filariasis CELISA

Throughout this thesis the Filariasis CELISA anti-Bm14 IgG_4 assay is referred to as "Filariasis CELISA". Sample diluent was prepared according to the manufacturer's instructions (Appendix 1) and 500 µL was transferred into separate plastic 1 mL tubes using a micropipette. A single protrusion of blood soaked onto filter paper was excised into the tube using scissors and vortexed (Figure 3.3). As the disc contains 10 µL of blood, it is assumed that this represents 5 µL of serum and thus a 1:100 dilution. The tube was left to elute overnight at 4^{o}C. The following day, eluates were warmed to ambient temperature and vortexed before commencing the assay.

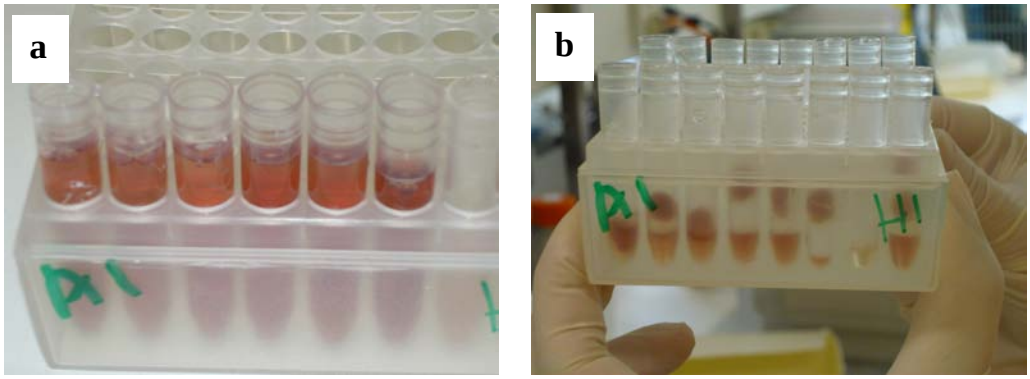


Figure 3.3: Elution of filter paper samples in preparation for the Filariasis CELISA.

(a) Following addition of sample diluent to the tube containing the filter paper the tubes were thoroughly vortexed. (b) Filter papers within plastic tubes.

3.2.3.2 The Og4C3 antigen assay

Sample preparation was in accordance with the manufacturer's instructions (Appendix 2). Two hundred microliters of sample diluent was added to each plastic tube contained within a 96-tube box with holes (Figure 3.4). Three protrusions from each filter disc were placed into the plastic tubes and then thoroughly vortexed. This was equivalent to a 1:13 dilution. The tube was left overnight at 4^{o}C. The following morning the samples were boiled for 5 minutes in a water bath heated to 100^{o}C (Figure 3.4). Following boiling, the samples were centrifuged (2000 ***g***; 5 minutes) leaving any potential antigen in the supernatant for detection.

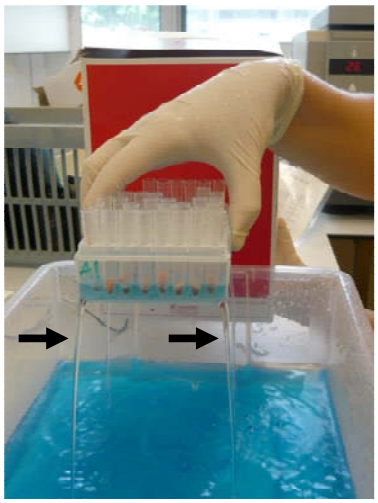


Figure 3.4: Puncturing the tube container to allow free flow of water for the boiling step in the Og4C3 assay. It is essential to have the correct water volume in the water bath during boiling so as not to overflow into the plastic tubes. The holes in the container allow entry of water.

3.2.4 Microfilarial (Mf) Detection

Due to field conditions and logistical difficulties, the thick blood smear was chosen as the method for Mf detection as previously described (Sasa, 1976). Collected blood (Section 3.2.2) was drawn into three lines, each containing approximately 20 µL, onto a microscope glass slide using a capillary tube (Figure 3.5). The slides were left to dry for 48 hours then wrapped for transport (Section 3.2.2). Each slide was stained in 10% Giemsa stain (20 minutes), washed in water, dried, and then coverslipped. The slide was examined under the microscope (x 200) and Mf were recorded. The number of Mf per mL of blood was calculated based on the initial 60 µL volume. In areas of nocturnal periodicity Mf samples were taken between 2200 hours and midnight in accordance with peak microfilaraemia (Abe ***et al.***, 2003). For areas where the diurnal sub-periodic form exists, where ***Aedes*** sp. are the main vector, samples were taken during daylight hours from 0800 hours to 2000 hours (Ramalingam and Belkin, 1964).

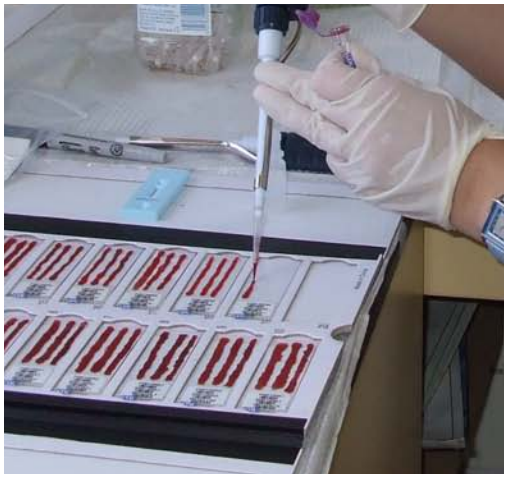


Figure 3.5: The thick blood smear for Mf detection.

3.2.5 Antigen Detection

3.2.5.1 NOW® filariasis Immunochromatographic test (ICT)

The ICT was performed in the field as previously described (Weil ***et al.***, 1997). Briefly, the collected 100 µL of blood was transferred onto the absorbent pad (Figure 3.6) and the test card was closed firmly. The result was read at exactly 10 minutes since, if left too long, a false positive may occur (Rajgor ***et al.***, 2002). A positive result was depicted as a line at both the control (C) and test (T) windows, whereas a negative result was only one line at the C window (Figure 3.6). The test was invalid if no line appeared.

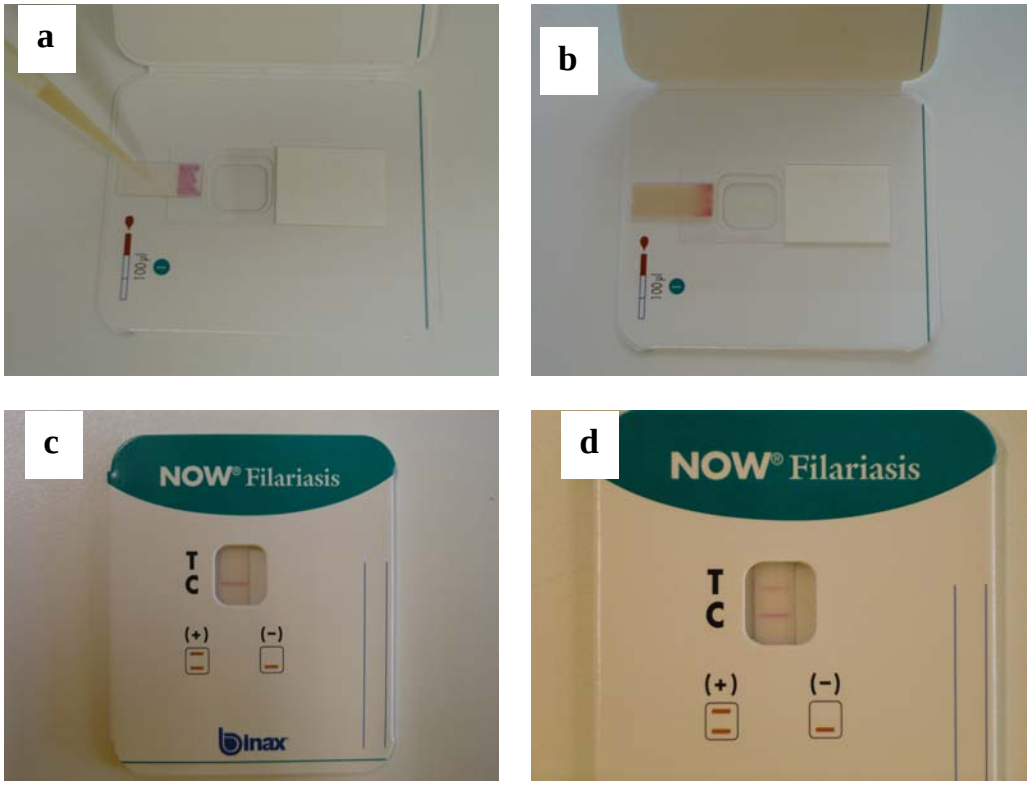


Figure 3.6: The ICT. (a) Adding 100 µL of blood or serum to the absorbent pad. (b) Following addition of the sample the card is sealed shut and the assay is read after 10 minutes. (c) A negative test is represented by a single line at the C in the viewing window. (d) A positive test is represented by a line at both the T and C in the viewing window.

3.2.5.2 The Og4C3 ELISA

The assay was performed according to the manufacturer's instructions (Appendix 2). Samples were tested in duplicate and plates were read at a dual wavelength of 414 nm and 492 nm with a Multiskan Ascent V1.24 spectrophotometer (Pathtec, Victoria (VIC), Australia) using the Ascent Software Version 2.6 (Pathtech, VIC, Australia). The absorbance value of standard three was considered the reactive cut-off. Therefore, any sample

with an Optical Density (OD) absorbance equal to or greater than this was considered positive.

3.2.6 Antibody Detection

Filarial antibodies were detected using the Filariasis CELISA according to the manufacturer's instructions (Appendix 1). Samples were tested in duplicate and reported throughout the thesis as "antibody positive" or "antibody negative" based on being reactive or non-reactive respectively. The washing steps were performed with an automated plate washer (MultiDrop® Combi nL, Pathtec, VIC, Australia) using 200 µL per well. Initially plates were read at a dual wavelength of 450 nm and 650 nm with a Multiskan EX Type 355 Primary V.2.1-0 (Pathtec, VIC, Australia) using the software Labsystems Genesis Version 3.00 (Pathtec, VIC, Australia). Subsequently, a new plate reader was used: a VersaMax PLUS ROM V1.21 (Molecular Devices, USA) with software SoftMax Pro Version 5.3 (Molecular Devices, USA). Experiments utilising the new spectrophotometer only included Sections 4.3.6 and 5.3.1.4. Initially plates were read simultaneously on both plate readers. OD absorbance values for the new plate reader were comparable to the old plate reader. The new software was more user-friendly.

3.3 STATISTICAL ANALYSIS

Methods used for statistical analysis is described in depth in each respective chapter. Overall most analyses were performed using the SPSS statistical software Version 17.0. Prevalence of diagnostic parameters was mapped using the geographic information system (GIS) package ArcGIS 9.3 (ESRI, 2008). The spatial statistical programme used was SaTScan (Kulldorff, 1997). Ninety five percent confidence intervals (95%-CI) were determined using the Binomial Stats programme "JavaStat" (Clopper and Pearson, 2005).

3.3.1 Normalisation of Data for SPSS Statistical Software Version 17.0

Normality assumptions were examined when using SPSS to assess if data were parametric or non-parametric as this would affect the statistic to use (Muller and Buttner, 2009). The data was considered to be normally distributed, and thus parametric, if all of the three following conditions were met:

- The histogram created from the data followed the shape of a Gauss bell (Figure 3.7).
- The ratio of the mean and the median was between 0.9 and 1.1.
- The ratio of the standard deviation (SD) and the mean was < 0.3.

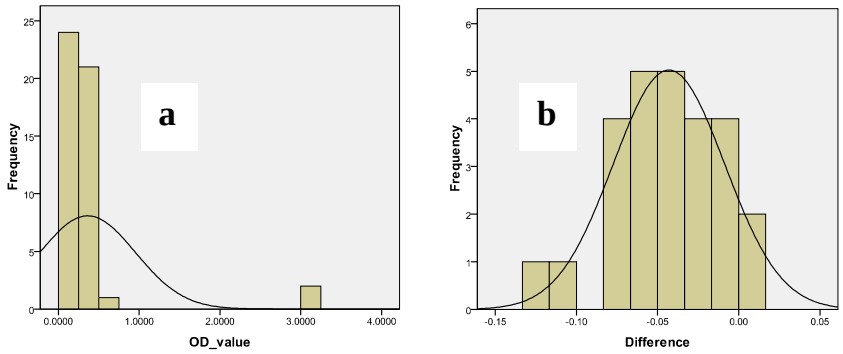


Figure 3.7: Shape of the histogram after the frequency of the data points were plotted using SPSS 17.0. (a) The data is non-parametric as the curve is skewed to the left. Therefore, only non-parametric statistical analyses can be used. (b) This is a normally distributed data set following a Gauss bell shape and therefore parametric analyses can be used.

3.4 ETHICS APPROVAL

The study was conducted under human ethics approval numbers H1423 and H2816, as approved by the JCU Research Human Ethics Committee (Appendices 3 and 4 respectively). For each field site, I sought prior approval from the relevant Ministries of Health and WHO representatives prior to conducting any research. Study protocols were also submitted and approved by the ministries. Blood collection from participants (Section 3.2.2) was conducted following informed verbal consent. The community survey (Chapter 8) was also included in H1423.

CHAPTER 4

THE VALIDITY OF THE FILARIASIS CELISA FOR USE AS A LYMPHATIC FILARIASIS DIAGNOSTIC ASSAY

4.1 INTRODUCTION

Although antibody assays for filarial nematodes are unable to distinguish between past or current infections, their application is useful since previous studies have demonstrated that antibody production in endemic areas occurs during the first few years of life (Gao ***et al.***, 1994; Lammie ***et al.***, 1998). Detection of antibodies as a diagnostic tool is useful for diagnosis of exposure to filarial nematodes for individuals residing in non-endemic areas, since a positive test would be indicative of exposure and guide decisions concerning treatment. In the past, antibody assays in endemic areas proved not to be as useful because the community was constantly exposed to infective mosquitoes and many individuals would be expected to be antibody positive. However, with the approach of the end of the MDA, the age prevalence of positive serology should have shifted; with younger individuals born after the MDA being antibody negative if no exposure to the parasite occurs (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). This now makes the antibody assay a potential tool for monitoring LF exposure for both endemic and non-endemic countries.

The optimal cohort for surveying in endemic countries would be children born after MDAs.

Antibody assays were developed as early as 1980 and a wide range of crude filarial parasite antigens and lysates were utilised (reviewed in Melrose, (2004)). The earlier assays based on crude parasite lysate were limited in terms of specificity because of their potential for cross-reaction with other parasitic infections (Maizels ***et al.***, 1985; Langy ***et al.***, 1998; Muck ***et al.***, 2003). This made their use in LF programmes inadequate since in LF endemic areas poly-parasitism is common (Utzinger and de Savigny, 2006).

The LF research community has witnessed the move from these earlier antibody assays, reliant on crude parasite lysates, to the recent more specific and sensitive recombinant antigen based antibody assays (reviewed in Melrose ***et al.***, (2004)). The advent of recombinant antigen detection systems has increased the specificity of the antibody assays by reducing cross-reaction with other parasitic infections (Lammie ***et al.***, 2004). This increases the potential of antibody serology to be used diagnostically in the LF programme. The recombinant antigens commercially available are the Bm14, SXP, and the BmR1 (Lammie ***et al.***, 2004) (Section 2.4.4.2).

The applicability of the recombinant antigens differs depending on the endemic country. BmR1 is a ***B. malayi*** recombinant antigen, which has been shown to react specifically with sera from ***B. malayi*** infected individuals and ***B. timori*** infected individuals (Rahmah ***et al.***, 2001a; Rahmah ***et al.***, 2001b;

Supali ***et al.***, 2004). However, assay specificity for ***W. bancrofti*** has been reported as quite low (Lammie ***et al.***, 2004). The use of the recombinant antigen Bm14, in the commercially available Filariasis CELISA combats this problem.

Bm14 is a 132 kDa recombinant antigen (Chandrashekar ***et al.***, 1994). Previous research utilising the antigen in the research-based Bm14 assay prototype demonstrated high sensitivity and specificity (Lammie ***et al.***, 2004). This research showed reactivity with sera from patients with Brugian or Bancroftian filariasis with reported sensitivities ranging from 90% to 96% (Lammie ***et al.***, 2004). Specificity studies were also promising as no cross reaction was reported with strongyloidiasis, schistosomiasis and other intestinal helminths (Chandrashekar ***et al.***, 1994; Lammie ***et al.***, 2004). Unfortunately, the assay reacted with 72% of the ***L. loa*** and ***O. volvulus*** positive sera, limiting assay usefulness in African regions (Lammie ***et al.***, 2004). Since these laboratory experiments, the research-based Bm14 assay prototype has been widely used in field studies with favourable results in terms of accurate detection of anti-filarial antibody (Li ***et al.***, 2004; Washington ***et al.***, 2004; Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Njenga ***et al.***, 2007a; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). There are two prominent disadvantages of these studies, by employing the assay prototype. No direct intra- or interlaboratory comparisons can be concluded since standardised reagents were not used nor were stringent QC practices enforced, such as that seen with commercially available reagent kits (Weil ***et al.***, 2010). The commercially available Filariasis CELISA, based

on this research-based Bm14 assay prototype, is yet to be studied. For the assay to be useful in terms of LF programmatic decisions, it must be easily adaptable for field surveys.

Wide-scale implementation of the Filariasis CELISA in the LF programme requires initial stringent laboratory experiments with serum. This is to ascertain that the new commercially available assay meets, and even surpasses, the original specifications of the research-based Bm14 assay prototype (Chandrashekar ***et al.***, 1994). This is especially critical since the commercially available assay differs in three main ways. The kit itself has a shelf-life of 12 months and is manufactured under "Good Manufacturing Practice" (GMP) conditions, with standardised reagents and QC, unlike the research-based Bm14 assay prototype (Weil ***et al.***, 2010). GMP conditions involve continual auditing of the manufacturer to ensure they are meeting the required international standards (ISO 13485:2003) enabling the assay to be used for both diagnostic and research purposes. Lastly, the kit is assumed to have higher sensitivity as the detection substrate system for the Filariasis CELISA uses a more reactive horseradish peroxidase (tetramethylbenzidine (TMB) versus (vs) ***o***-phenylenediamine (OPD)) (Weil ***et al.***, 2010). Preliminary studies, in which I was involved (Section 4.3.1.1), concluded that the commercial assay was robust, highly comparable among laboratories, and standardised (Weil ***et al.***, 2010), highlighting its potential use in the LF programme.

For successful implementation into the repertoire of LF diagnostic assays available to programme managers, the Filariasis CELISA must be easily adaptable for field surveys. Sample recommendations from the manufacturer include serum or plasma, yet paired samples drawn from endemic individuals have not been investigated for sample comparisons. Results with serum alone are promising (Weil ***et al.***, 2010). However, for any long term survey work an easier sampling method would be required since venepuncture, transportation and storage of serum can be difficult in endemic remote areas (Figure 4.1). Filter paper sampling from fingerprick blood is more cost-effective, easier, and there is limited sample variation due to fluctuations in temperature since specimens thoroughly dried can be stable at ambient temperature for up to a week (Coltorti ***et al.***, 1988). Previously, the filter paper method has been shown to be a suitable alternative for anti-filarial IgG_4 antibody assays based on crude protein lysate and ELISA antigen assays such as the Og4C3 (Terhell ***et al.***, 1996; Gyapong ***et al.***, 1998; Itoh ***et al.***, 1998; Hoti ***et al.***, 2002). In a controlled laboratory experiment, using spiked samples soaked onto filter paper, sample reactivity between serum and filter paper samples was comparable using the Filariasis CELISA (Weil ***et al.***, 2010). The authors concluded that further studies were required in a field setting. Field filter paper studies for the Filariasis CELISA are yet to be conducted.

The aim of the research outlined in this chapter was to assess the suitability of the Filariasis CELISA assay as a potential tool for monitoring the elimination of LF. To achieve this, initial laboratory work with serum samples

was required to assess comparability to the research-based Bm14 assay prototype. Secondly, optimisation of filter paper sampling was required. Thirdly, stability of kit reagents was assessed since implementation of the Filariasis CELISA into the available repertoire of diagnostic assays would require long transit for overseas shipment. Key findings from this research chapter have been published (Joseph and Melrose, 2010).

Figure 4.1: LF Field Survey: Vanuatu April 2008. Field collection can often be confounded by remoteness of village sites, often accessible only by boat. Transportation of supplies, and storage of collected specimens, can therefore be logistically difficult.

4.2 AIMS

The specific aims for the work described in this chapter are to:-

(1) Determine the specificity of the Filariasis CELISA and assess cross-reactivity with ***Strongyloides*** sp. reactive serum;

(2) Assess the validity of the manufacturer's suggested serum dilution of 1:100 using known reactive sera from a highly endemic LF area in PNG;

(3) Optimise the Filariasis CELISA for filter paper sampling;

(4) Assess the sensitivity and specificity of the Filariasis CELISA with filter paper samples;

(5) Assess the durability of the reagents of the Filariasis CELISA kits following delayed transportation; and,

(6) Assess the longevity of filter paper sample storage at -20^{o}C for 10 months.

4.3 MATERIALS AND METHODS

4.3.1 Cross Reactivity of the Filariasis CELISA With Other Parasites

4.3.1.1 Multicentre evaluation

As part of a blinded study, 81 coded serum samples were sent to JCU from Dr Gary Weil (Associate Professor of Molecular Microbiology, Washington University Laboratory, Saint Louis, USA). Upon arrival, serum was stored at -20^{o}C until tested. Other research laboratories involved included Bernard Nocht Institute (Germany) and the CDC (Atlanta, USA). I never had access to the details of the 81 coded sera.

Samples were prepared by diluting serum 1:100 with supplied sample diluent as per manufacturer's instructions (Appendix 1) and thoroughly vortexing. The Filariasis CELISA was performed in accordance with the manufacturer's instructions and OD absorbance values measured (Appendix 1; Section 3.2.6) with the following exceptions. A reactive sample was defined

as obtaining an OD absorbance value of ≥ 0.250. Samples with an indeterminate OD value between 0.180 and 0.250 were retested and if the OD value was still < 0.250, samples were considered non-reactive. Results were reported back to Dr Gary Weil for manuscript submission and publication (Weil ***et al.***, 2010).

4.3.1.2 Cross reactivity with ***Strongyloides*** sp. sera

Twenty serum samples that were positive for ***Strongyloides*** sp. antibodies were kindly donated by Dr Rogan Lee (Centre for Infectious Diseases and Microbiology Lab Services, Westmead hospital, Sydney). These samples were tested for ***Strongyloides*** sp. antibodies in Dr Lee's laboratory based on somatic whole antigen. The samples originated from migrants to Australia, although strongyloidiasis is endemic in Australia (Sampson and Grove, 1987). Upon arrival, the sera were stored at -20^{o}C until tested and samples were prepared as previously described (Section 4.3.1.1). Following preparation, diluted samples were run in duplicate using the Filariasis CELISA according to manufacturer's instructions (Appendix 1) and OD absorbance values were measured (Section 3.2.6). Specificity was determined using the Chi-squared test with the software SPSS Version 17.0.

4.3.2 Optimisation of Serum Sample Dilution

Previously collected sera (n = 90) from PNG, with known Mf and CFA status (Melrose, 2002), were used to confirm the manufacturer's recommended test dilution of 1:100. Samples were chosen randomly from the serum bank irrespective of age, gender, and infection status. Three dilutions, with sample diluent, were chosen (1:100, 1:500 and 1:1000). Antibody titres of these serum samples had been previously assayed utilising a research-based ELISA with crude ***Dirofilaria immitis*** antigen extract and recorded as units, not OD absorbance values (Melrose, 2002). Thus, sample reactivity could be compared between the two assays, but not serum levels of antibody. Following preparation, samples were run in duplicate using the Filariasis CELISA and OD absorbance values were measured (Appendix 1; Section 3.2.6). OD absorbance values were plotted for each serum sample titration. Chi-squared analysis was used to compare the sensitivity and specificity of the new Filariasis CELISA with the previously used ***D. immitis*** research assay (n = 84).

The serum databank contained information regarding the LF status of each individual, although this was not a prerequisite for inclusion in the present study (Table 4.1). The obtained antibody results from the present study were investigated in terms of LF status (Mf or CFA) using the Chi-squared statistic (n = 82). OD absorbance values were assumed to correlate with serum levels of antibody (Dylewski ***et al.***, 1984). This was achieved using the

Kruskall-Wallis Test for non-parametric data following checking for normality (n = 82) (Section 3.3.1).

Table 4.1: Demographic information of samples derived from the PNG Serum Bank. There was a similar spread of age range for males and females. Interestingly, one male tested positive for Mf, but was CFA negative. This one sample was excluded from statistical analysis.

Characteristic	**Male**	**Female**	**TOTAL TESTED**
Median age, years	13 ½	16	
Age range, years	1 - 70	1 – 60	
LF status			
Mf positive CFA positive	8	7	15
Mf positive CFA negative	0	0	0
Mf negative CFA positive	4	15	19
Mf negative CFA negative	18	30	48
TOTAL TESTED	30	52	**82**

4.3.3 Optimisation of Filter Paper Sampling for the Filariasis CELISA

4.3.3.1 Negative control

Controls were supplied with the Filariasis CELISA. Negative cut-offs are recommended by the manufacturer to be OD absorbance value ≤ 0.200 or the current negative control + 3SD. It was assessed whether this cut-off value for non-reactivity was still suitable for samples eluted from filter paper.

Following informed consent, 45 healthy volunteers with no history of LF exposure, or travel to LF endemic countries, were chosen to participate. Fingerprick blood samples were taken (Section 3.2.2) and prior to testing, samples were eluted (Section 3.2.3.1). The resulting eluate (containing approximately 1:100 dilution of sera) was tested in the Filariasis CELISA in duplicate and OD absorbance values were measured (Appendix 1; Section 3.2.6). The remaining blood spots were transported to Cellabs for validation. Cellabs is the laboratory which manufactures the Filariasis CELISA (Manly, NSW). These values were included in establishing the negative cut-off for filter paper samples. The negative cut-off was defined as the average + 3SD.

4.3.3.2 Reducing background signal to noise ratio

Thirty four school children aged between 6 and 8 years from the southern island of ‘Eua in Tonga were chosen for this aspect of the study. These children attended the primary schools Ha’atu’a, Angaha and Ohonua. Sera samples from these individuals were either antibody positive previously or close to the OD absorbance cut-off value of 0.400 (Chapter 5). Written consent from their parents was obtained to take part in the study. Filter paper blood samples were collected (Section 3.2.2) and prior to testing samples were eluted (Section 3.2.3.1).

4.3.3.2.1 Blocking buffer

Half of the 96-well ELISA plate was incubated with blocking buffer (Phosphate Buffered Saline (PBS) pH 7.4, 5% skim milk, 0.05% Tween-20), 200 uL per well for one hour at ambient temperature prior to testing. Following incubation, the plate was washed four x with the manufacturer's washing buffer and the assay was then performed as previously described (Appendix 1; Section 3.2.6). Twenty-one of the 34 Tongan eluates were tested in duplicate on both sides of the plate for direct method comparison (blocking buffer vs. no blocking buffer). OD absorbance values were statistically compared using the paired Wilcoxon test for non-parametric data (SPSS Version 17.0).

4.3.3.2.2 Hydrogen peroxide

The initial ELISA steps were performed (Appendix 1) and 13 of the 34 Tongan eluates were run in duplicate on both sides of the ELISA plate. Following sample incubation, and plate washing, half of the 96-well ELISA plate was incubated with peroxidase blocking buffer for 10 minutes at ambient temperature in the dark and then washed four x in supplied washing buffer. Peroxidase blocking buffer consisted of a 3% hydrogen peroxide (H_2O_2) solution prepared in sample diluent. The rest of the ELISA was performed according to the manufacturer's protocol (Appendix 1; Section 3.2.6). OD absorbance values were statistically

compared using the paired Wilcoxon test for non-parametric data (SPSS Version 17.0).

4.3.4 Sensitivity and Specificity of Filter Paper Sampling

In the laboratory 94 samples were selected, blinded to disease and demographic status, from specimens remaining from a cross-sectional survey of 1500 people in Tuvalu. Verbal consent was given and approximately 200 µL of blood was collected using the fingerprick method by capillary action into ethylenediaminetetraacetic acid (EDTA) vacutainers (Becton, Dickinson and Company (BD), North Ryde, NSW). Following collection, six x 10 µL blood was blotted onto filter paper, dried, transported and stored as described (Section 3.2.2). The remaining blood in the vacutainer was left overnight at 4^{o}C to allow the red cells to settle as centrifugation was not available. The following morning the plasma was aliquoted into a fresh sterile tube, stored at 4^{o}C and transported at 4^{o}C to Australia. Upon arrival in the JCU laboratory, both filter papers and plasma were stored at -20^{o}C until tested. Samples were eluted from the filter papers the day prior to testing (Section 3.2.3.1). The plasma samples were prepared the day of testing by diluting 5 µL into 495 µL of sample diluent (1:100).

Each paired eluate and diluted plasma sample were tested in duplicate according to the manufacturer's instructions (Appendix 1; Section 3.2.3.1; Section 3.2.6). Filter paper reactivity was defined as OD reading ≥ 0.400

whereas serum reactivity was defined as OD reading ≥ 0.250 based on previous research (Weil ***et al.***, 2010). Sensitivity and specificity between the two methods was determined using the Chi-squared test whereas OD absorbance values were compared using the paired Wilcoxon test (SPSS Version 17.0).

4.3.5 Kit Durability Following Delayed Transportation

Five Filariasis CELISA kits, each containing five x 96-well coated plates, were sent from the manufacturer by road transport (approximately 2100 kms) and received five days later. Despite transportation of kits with frozen ice packs, the package contents had warmed to ambient temperature by the time of receipt. Subsequently, the kits were immediately transferred to 4^{o}C for storage. Another five Filariasis CELISA kits were transported by air travel and were received the following day, still cool, and immediately transferred to 4^{o}C for storage. Therefore, it was assumed that the former kits were subjected to approximately three days at ambient temperature. To assess whether the kit components were still stable after a long travel time, the positive control was titrated in doubling dilutions from 1:100 to 1:3200 on a randomly chosen plate from each kit. Testing was in duplicate for both the road and air transport ELISA plates. OD absorbance values were compared using agreement statistics based on a scatter plot (SPSS Version 17.0). From the scatter plot, a concordance correlation coefficient was determined, which measures the agreement between two variables (Lin, 1989; Dewe, 2009).

4.3.6 Effect of Storage Temperature on Reactivity of Filter Paper Samples

Following verbal consent, 495 participants in the village of Siufaga in Samoa were screened for antibodies using the Filariasis CELISA. Screening was performed using the filter paper technique (Section 3.2.3.1; Section 3.2.6). Based on these preliminary findings (Chapter 6), 200 samples were chosen for storage at -20^{O}C for 10 months and re-tested. The 200 samples chosen were based on the initial OD absorbance value. One hundred and one of the chosen samples were reactive, where 50 samples were high reactors with an OD absorbance value > 1.1. The remaining 51 of the reactive samples had lower OD absorbance values ranging from 0.400 to 0.611. The 99 non-reactive samples chosen ranged from OD absorbance values of 0.013 to 0.354. Fifty one of the non-reactive samples had initial OD absorbance values close to the positive cut-off value of ≥ 0.400 in order to determine if after storage these non-reactive samples became false positives. Results, including OD absorbance values, were compared between the two time periods using both the chi-square analysis and the Mann-Whitney ***U*** test (SPSS Version 17.0).

4.4 RESULTS

4.4.1 Cross Reactivity of the Filariasis CELISA With Other Parasites

4.4.1.1 Multicentre evaluation

Results of the multicentre evaluation, with myself as a co-author, have been published (Weil ***et al.***, 2010). Results showed minimal interlaboratory variation and high assay specificity. Specificity studies included in the publication were also derived from my own work (Section 4.4.1.2).

4.4.1.2 Cross reactivity with ***Strongyloides*** sp. sera

Of the twenty ***Strongyloides sp*** antibody positive sera, only two were reactive (Figure 4.2). Re-evaluating the data using the flexible definition of a reactive sample by Weil ***et al***. (2010) (OD ≥ 0.250) did not alter the results. Upon further investigation it was discovered that the two reactive samples were drawn from the same individual. Therefore, out of 19 serum samples, only one was reactive resulting in a specificity of 94.7%.

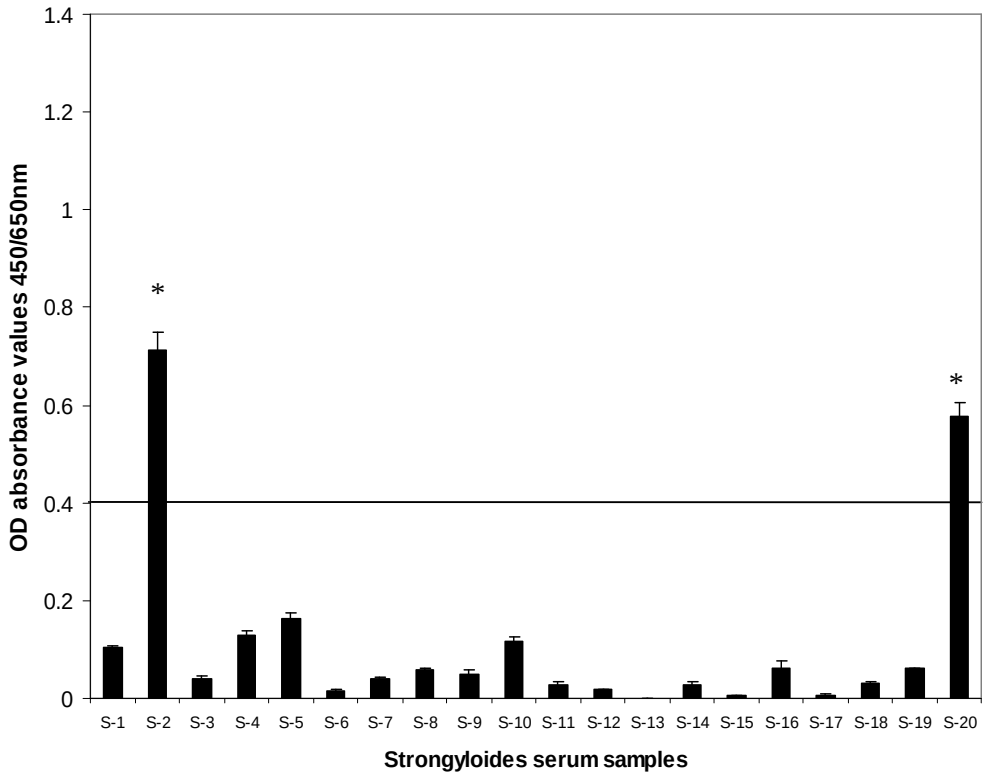


Figure 4.2: **OD absorbance values for 20 sera obtained from individuals positive for anti-*Strongyloides* sp. antibodies as measured using the Filariasis CELISA.** Eighteen samples were below the positive cut-off and were non-reactive in the ELISA. Two samples, both from the same individual, were reactive (*), giving the Filariasis CELISA for serum samples an overall specificity of 94.7% (1/19) (95%-CI = 74.0 – 99.9).

4.4.2 Optimisation of Serum Sample Dilution

As expected, the majority of the 90 PNG sera samples were reactive with the Filariasis CELISA and the level of reactivity decreased when the sample was titrated (Figure 4.3; Appendix 5).

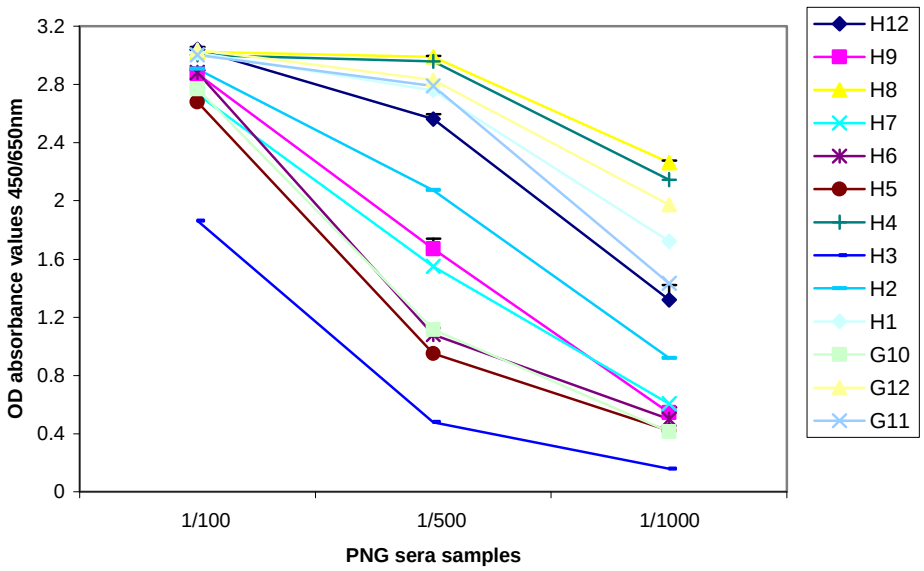


Figure 4.3: OD absorbance values for 13 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased. The results for the all sera are located in Appendix 5.

The Filariasis CELISA had higher sensitivity than the anti-filarial IgG_4 ***D. immitis*** laboratory ELISA (Table 4.2). The earlier assay, in comparison to the Filariasis CELISA, had a low sensitivity of 39.3% and negative predictive value (NPV) of 27.5%.

Table 4.2: Cross-tabulation results for detection of anti-filarial IgG_4 comparing the new commercial Filariasis CELISA with previous research-based *D. immitis* ELISA. The gold standard was considered to be the new commercial assay since it is based on recombinant antigen (columns). ELISA specifications for the earlier ***D. immitis*** ELISA was found to be a sensitivity of 39.3% (95%-CI = 27.1 – 52.7), specificity of 60.9% (95%-CI = 38.5 – 80.3), Positive Predictive Value (PPV) of 72.7% (95%-CI = 54.5 – 86.7), and NPV of 27.5% (95%-CI = 15.9 – 41.7).

		Filariasis CELISA		
		Negative	Positive	TOTAL
D. immitis ELISA	Negative	14	37	51
	NPV	27.5% (14/51)		
	Specificity	60.9% (14/23)		
	Positive	9	24	33
	PPV		72.7% (24/33)	
	Sensitivity		39.3% (24/61)	
	TOTAL	23	61	84

The PNG sera samples included information on Mf and CFA status (Melrose, 2002) (Table 4.1). Although amicrofilaraemic CFA positive individuals had the highest number of antibody reactors (78.9%), there were no significant differences among the clinical groups for antibody status (Figure 4.4; P = 0.705).

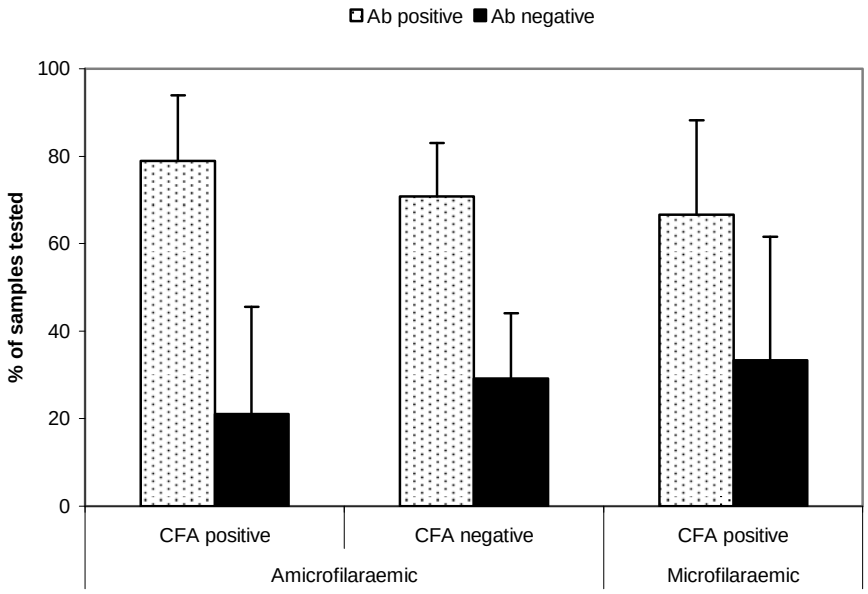


Figure 4.4: Antibody (Ab) positivity amongst individuals from PNG where there is high intensity of LF transmission. The category of amicrofilaraemic and CFA positive individuals had the highest number of reactors with 78.9% (95%-CI 54.4 – 94.0) of sera containing antibodies. The Mf and CFA positive individuals only had 66.6% (95%-CI 38.4 – 88.2) of individuals with antibodies, whereas the endemic normals (Mf and CFA negative) had 70.8% (95%-CI 55.9 – 83.1). There were no significant differences among the clinical groups for antibodies (χ^2 = 0.698; degrees of freedom (df) = 2; P = 0.705).

OD absorbance values were assumed to equate to serum levels antibody (Dylewski ***et al.***, 1984). Although the average OD absorbance value for Mf and CFA positive samples was higher (1.928) than the Mf negative and CFA

positive (1.844) and Mf negative and CFA negative (1.568) individuals, this was not significant (χ^2 = 1.321; df = 2; P = 0.517).

4.4.3 Optimisation of Filter Paper Sampling for the Filariasis CELISA

4.4.3.1 Negative control

The average OD absorbance values obtained for the 45 non-endemic eluted whole blood samples from filter paper from JCU and Cellabs were 0.08 and 0.13, respectively (Figure 4.5). To determine the negative cut-off value, the average of all 90 values + 3SD was used. Consequently, the negative cut-off absorbance value, when using eluted blood samples in the Filariasis CELISA, was set at OD ≤ 0.260.

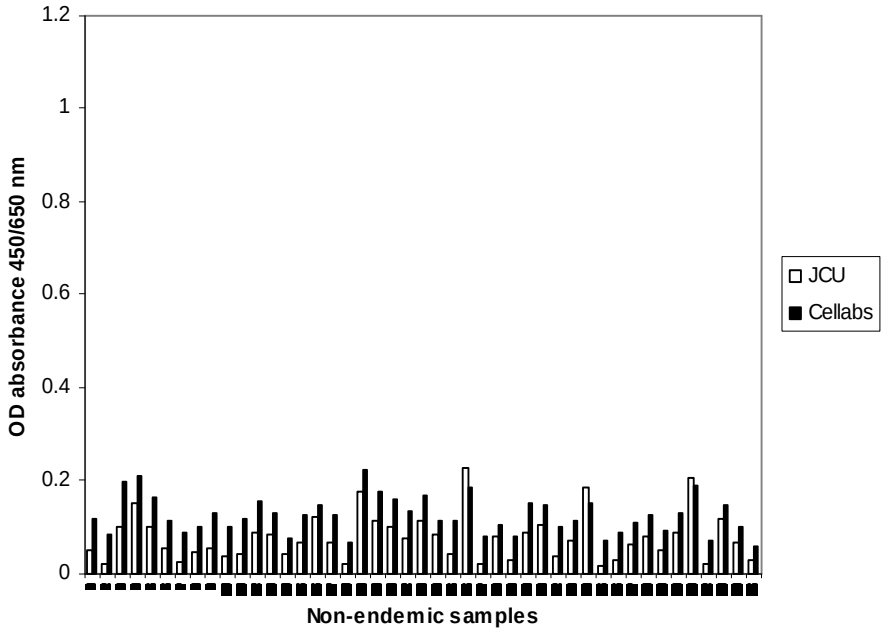


Figure 4.5: Negative cut-off values of eluates from filter paper samples as measured using the Filariasis CELISA. Negative cut-off values for the Filariasis CELISA, when testing for antibodies in whole blood eluted from filter paper, were obtained using 45 healthy volunteers with no history of LF or travel to LF endemic countries. Two separate laboratories assayed the samples and the average + 3SD of the reactivity of the non-endemic samples was calculated as OD ≤ 0.260.

4.4.3.2 Reducing background signal to noise ratio

4.4.3.2.1 Blocking buffer

The difference in OD absorbance values between the two methods (blocking buffer vs. no blocking buffer) was calculated. Reduction of OD absorbance values following blocking was depicted as a positive value (white) (Figure 4.6). If the OD absorbance value increased after blocking, the data was represented as a negative value (black) (Figure 4.6). There was no

statistical significance between the two treatment groups using the paired Wilcoxon test for non-parametric data (P = 0.199).

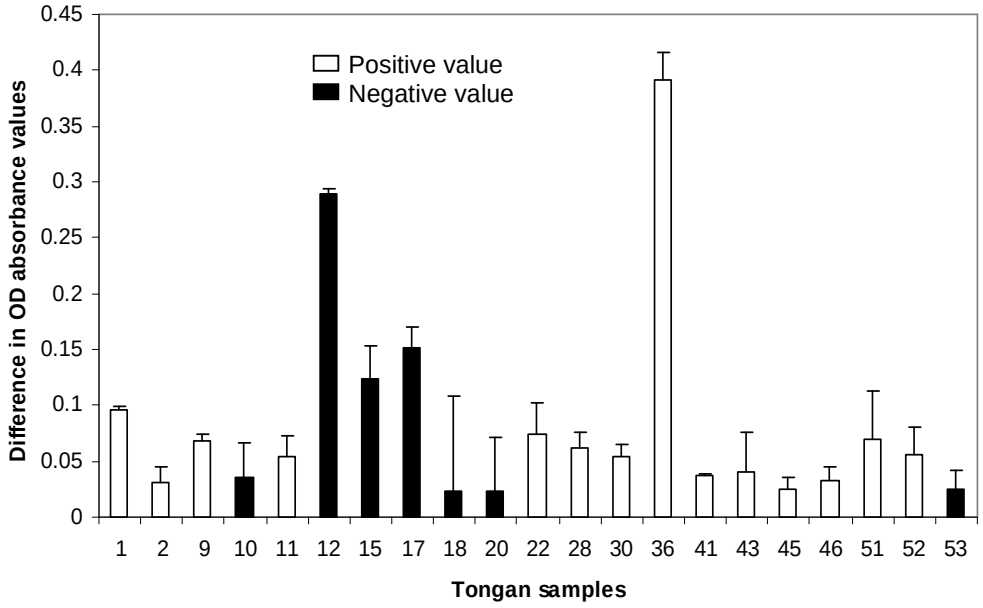


Figure 4.6: The difference in OD absorbance values of Tongan samples assayed by the Filariasis CELISA; comparing the standard protocol with the addition of a blocking step. A positive value indicates that there was a reduction in absorbance with the addition of the blocking step (white). A negative value indicates that following the blocking step the absorbance value was actually higher (black). There was no significant difference between the two methods (Z = -1.286; P = 0.199).

4.4.3.2.2 Hydrogen peroxide

The difference in OD absorbance values between the two methods was calculated (incubation with H_2O_2 vs. no H_2O_2). Again a reduction in OD absorbance values following treatment is depicted as a positive value (white bars) and an increase in absorbance values as a negative value (black

bars) (Figure 4.7). It was found that incubating the plate with H_2O_2 following sample incubation significantly decreased OD absorbance values using the paired Wilcoxon test for non-parametric data (P = 0). Despite the significant reduction in OD absorbance values, this did not affect whether a sample was considered reactive.

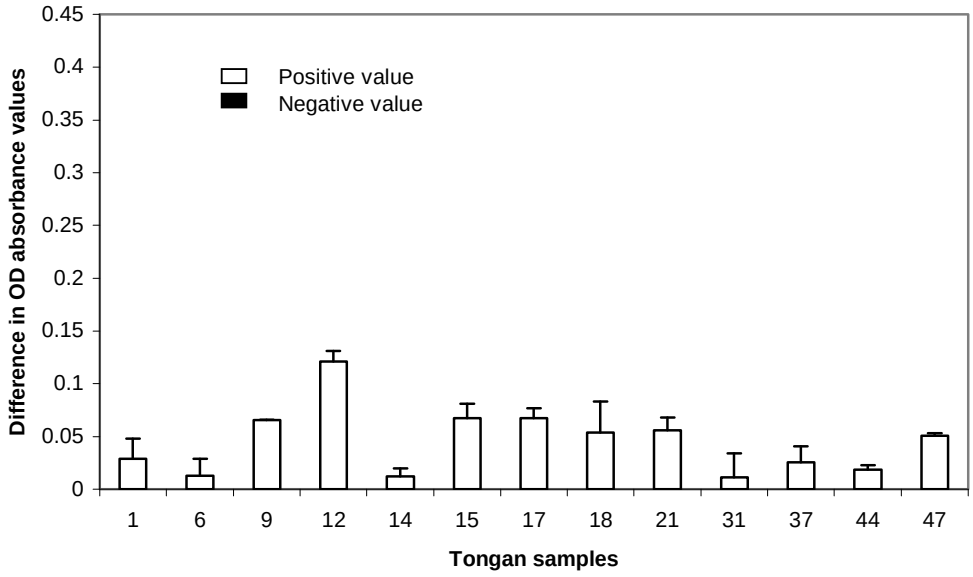


Figure 4.7: The difference in OD absorbance values of Tongan samples assayed by the Filariasis CELISA; comparing the standard protocol with the addition of incubation with H_2O_2. When the ELISA plate was treated with H_2O_2 following sample incubation, the OD absorbance value significantly decreased (Z = -4.305; P = 0).

4.4.4 Sensitivity and Specificity of Filter Paper Sampling

The sensitivity, specificity, Positive Predictive Value (PPV) and NPV for filter paper sampling, in comparison to plasma, is summarised in Table 4.3. The filter paper technique had a sensitivity of 92%, specificity of 77%, PPV of 60% and a NPV of 96%. There was a significant correlation between the two

methods of sampling using the Kappa agreement statistic (***r*** = 0.614; P < 0.001), although there was a significantly higher average OD absorbance value for filter paper samples (0.550) than serum (0.401) using the paired Wilcoxon statistic (Z = 6.273; P < 0.001).

Table 4.3: Cross-tabulation results for the Filariasis CELISA comparing paired plasma and filter paper samples. ELISA specifications for a filter paper eluate were found to be sensitivity 92.3% (95%-CI = 74.9 – 99.1), specificity 76.5% (95%-CI = 64.6 – 85.9), PPV = 60.0% (95%-CI = 43.3 – 75.1), and NPV = 96.3% (95%-CI = 87.3 – 99.6).

		Plasma		
		Negative	Positive	TOTAL
Filter paper	Negative	52	2	54
	NPV	96.3% (52/54)		
	Specificity	76.5% (52/68)		
	Positive	16	24	40
	PPV		60.0% (24/40)	
	Sensitivity		92.3% (24/26)	
	TOTAL	68	26	94

4.4.5 Kit Durability Following Delayed Transportation

Titration of the positive control was compared between the two batches of kits delivered by road and air (Figure 4.8). The two sets of OD absorbance values were plotted on a scatter plot, with a line of equality, to determine the spread of data around equality (Figure 4.9). The concordance correlation coefficient (Lin, 1989) was determined from the scatter plot to be 87.3%.

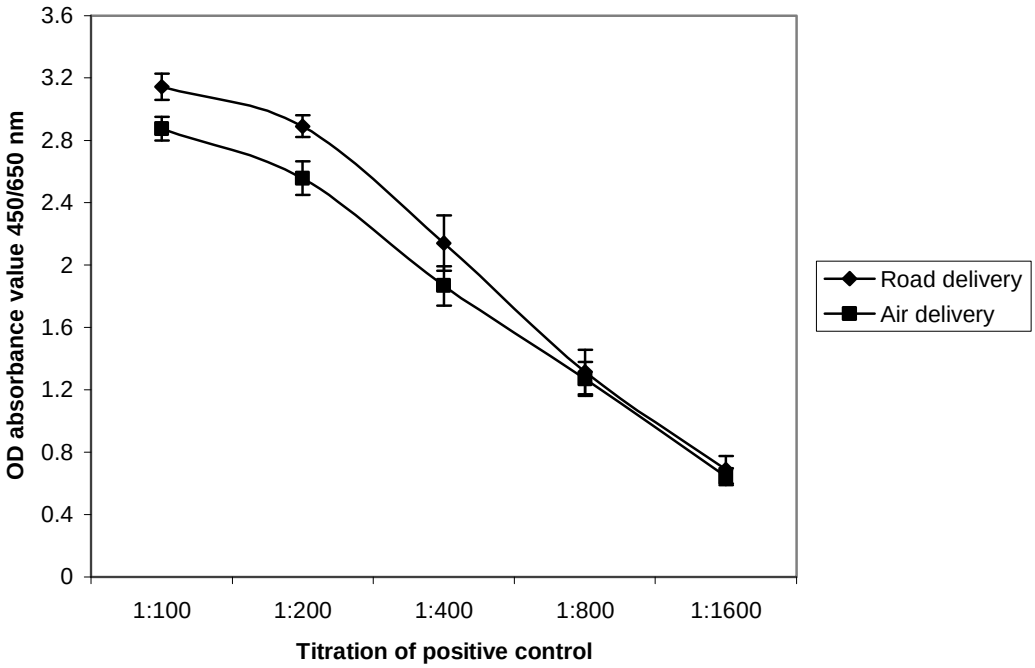


Figure 4.8: Comparison between two batches of kits received by different methods of transport (road and air); using titration of the positive control. Both sets of kits had similar values for the titration.

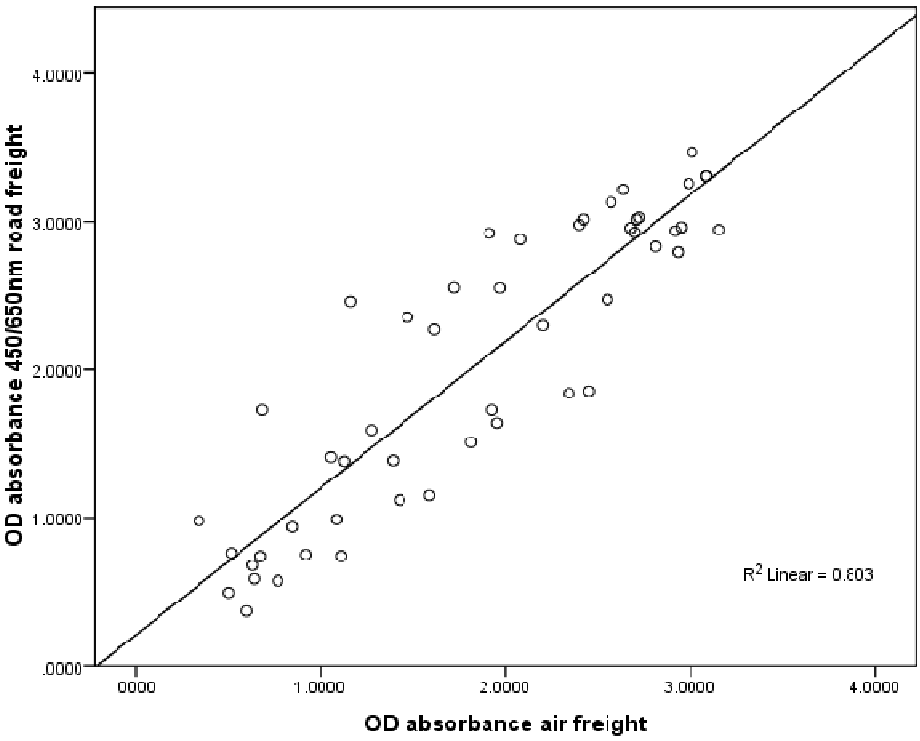


Figure 4.9: Scatter plot demonstrating the agreement relationship between the two batches of kits; one delivered by road (five days) and one by air (two days). The scatter plot, together with the line of equality, showed that the OD absorbance values obtained for the positive control titration using both batches of kits agreed quite well. From this the concordance correlation coefficient was calculated to be 87.3% (95%-CI 75.2 – 93.7).

4.4.6 Effect of Storage Temperature on Reactivity of Filter Paper Samples

The initial maximum and minimum OD values of the filter paper samples, prior to storage, were 3.9 and 0.013 respectively, with a median of 0.415. Following 10 months of storage at -20^{o}C, the OD maximum and minimum values decreased to 3.5 and 0 respectively, with a median reduction to 0.08.

This loss of reactivity was significant according to the Mann-Whitney U test ($P < 0.001$).

After storage, 67 of the original 101 reactive samples reduced below the reactivity cut-off value of ≥ 0.400 and were deemed non-reactive (Table 4.4). Thirty-four of the original 101 reactive samples remained reactive following storage. All of the 99 non-reactive samples remained non-reactive.

Table 4.4: Cross-tabulation results for the Filariasis CELISA comparing reactivity from paired filter paper samples following 10 months storage at -20^{o}C. Following storage of filter paper samples for 10 months, the reactivity of the samples significantly reduced (Z = 10.9; P < 0.001). Sixty-seven samples became non-reactive following storage, decreasing the sensitivity to 33.6% (95%-CI 24.6 – 43.8) and the NPV to 59.6% (95%-CI 51.8 – 67.2). Specificity (100%) and the PPV (100%) remained unchanged following storage (95%-CI 96.3 – 100 and 95%-CI 89.7 – 100 respectively).

		Initial Result		
		Negative	Positive	TOTAL
Following storage	Negative	99	67	166
	NPV	59.6% (99/166)		
	Specificity	100% (99/99)		
	Positive	0	34	34
	PPV		100% (34/34)	
	Sensitivity		33.6% (34/101)	
	TOTAL	99	101	200

4.5 DISCUSSION

The feasibility of incorporating serological testing into the LF programme depends not only on the accuracy of the assay, but also the ease of testing. The commercially available Filariasis CELISA was extensively optimised prior to manufacture and its research-based Bm14 assay prototype was applied in field studies with favourable results (Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). Since its availability from 2006, the assay is yet to be applied for large-scale sampling. The manufacturing process undergoes stringent QC checks in order to satisfy GMP and international standards, enabling standardisation (Weil ***et al.***, 2010). The commercial assay differs slightly from the assay prototype, and thus the efficacy of the assay needs to be initially validated under controlled laboratory conditions prior to adaptation for field studies.

As the Filariasis CELISA is part of the GMP, potential for inter-laboratory variation needed to be explored. I was included in a worldwide research study, encompassing three continents, whereby researchers were given a panel of unknown serum samples for testing. Despite different testing centres being involved, with different equipment, there was little interlaboratory variation. The low molecular weight recombinant antigen-based ELISA had a sensitivity of 98% and specificity of 92% (Weil ***et al.***, 2010). In onchocerciasis, serodiagnostic tests performed better when using low molecular weight antigen with patient's sera (Cabrera ***et al.***, 1988; Weiss and Karam, 1989; Bradley ***et al.***, 1991; Lobos ***et al.***, 1991). Conclusions

drawn from Weil ***et al***. (2010) were that the Filariasis CELISA reagents were stable and the assay had potential as a diagnostic tool for the detection of antibodies using serum. My cross-reactivity studies with ***Strongyloides*** sp. reactive sera were included in this publication.

Strongyloides sp. and other soil-transmitted helminths are usually co-endemic with LF (Malhotra ***et al.***, 1997; Padmasiri ***et al.***, 2006). Previous assays with crude lysate reported cross-reactivity with other parasites when assaying for anti-filarial antibodies (Abdullah ***et al.***, 1993; Muck ***et al.***, 2003; Riyong ***et al.***, 2003). Cross-reaction with Bm14 appears to be minimal using the research-based Bm14 assay prototype (Lammie ***et al.***, 2004). The results presented here concur with previous research since a high specificity of 94.7% was achieved with the commercial assay. Altering the reactive cut-off value from OD ≥ 0.400 to OD ≥ 0.250, as previously suggested (Weil ***et al.***, 2010), did not alter the specificity of the assay with the current dataset. The serum samples that were reactive in the assay were further investigated. It was established that both serum samples were drawn from the same individual, highlighting the reproducibility of the assay. The individual resided in Lebanon prior to emigrating to Australia. Unfortunately, there was no further access to previous history to determine if the individual had ever visited a LF endemic country. If so, it would have increased the specificity of the assay. However overall, the assay achieved high specificity (94.7%). These results agreed with previous studies using the research-based Bm14 assay prototype (Lammie ***et al.***, 2004), as well as the recent study using the Filariasis CELISA (Weil ***et al.***, 2010). IgG_4 detection in onchocerciasis has

also been shown to have limited cross reaction with other helminth infections, including ascariasis, hookworm, and strongyloidiasis (Weil ***et al.***, 1990).

Correct dilution of the sample is of critical importance since it can affect the sensitivity of the assay. This is linked to the antibodies avidity and/or affinity to the antigen coated on the plate (Lehtonen and Eerola, 1982). The current recommended dilution for the commercial assay is 1:100. If there are differences in affinity of antibody binding, when a low dilution of serum sample is tested (i.e. potentially high antibody concentration) then only those with high affinity will bind to the plate (Lehtonen and Eerola, 1982). An even mix of high and low affinity antibodies will begin to bind as the dilution increases, until reaching a saturation point. Based on this principle, if the recommended 1:100 dilution from the manufacturer is incorrect, the dose-response curve may follow a convex pattern, where only high affinity antibodies are able to bind at low serum dilutions. To test this theory, samples were chosen from an area of high LF endemnicity in PNG (Melrose, 2002) and thus potentially had high titres of antibodies. The results demonstrated a dose-response curve that decreased steadily following doubling dilutions. It was concluded that 1:100 sample dilution was unaffected by avidity or affinity of the IgG_4. Using this sample dilution would not interfere with specificity since the cross reaction studies at 1:100 were also favourable. The controls were also placed at different areas of the plate to avoid the edge effect, where there can be different heating of the plate in the incubator (De'Ath, 1988). Thus the commercial Filariasis CELISA had

comparable specificities and sensitivities for antibody detection and sampling at 1:100 was adequate.

In order to further explore sensitivity of the commercial assay, the reactivity of the PNG samples was compared to previous results obtained using the crude ***D. immitis*** antigen ELISA (Melrose, 2002). Since the commercial assay is based on a more sensitive and specific recombinant antigen (Lammie ***et al.***, 2004), this was considered the gold standard. Poor sensitivity (39.3%) was observed using ***D. immitis*** ELISA coupled with a low NPV of 27.5%. The low NPV suggests a false negative rate of over 70%, which would be unacceptable in programmatic applications. This reiterates the importance of using recombinant antigen ELISAs for their superiority over crude antigen-based ELISAs (Chandrashekar ***et al.***, 1994; Chandrashekar ***et al.***, 1996; Muck ***et al.***, 2003; Lammie ***et al.***, 2004; Li ***et al.***, 2004; Rahmah ***et al.***, 2004).

The PNG samples also included important information regarding the LF status of the patient. The antibody reactivity within each clinical group was investigated with no significant differences. It was observed that the highest number of reactors (78.9%) belonged to the amicrofilaraemic/CFA positive individuals. Interestingly, the lowest number of reactors (66.6%) were the Mf/CFA positive individuals. This conflicts with earlier studies using the research-based Bm14 assay prototype, as 80-90% of Mf positive individuals and only 37.6% of amicrofilaraemic/CFA negative individuals were antibody positive (Ramzy ***et al.***, 1995; Weil ***et al.***, 1999; Weil ***et al.***, 2008).

Lack of concordance could be due to a number of factors. Actual levels of anti-filarial antibodies in the adult population can vary depending on the previous history of LF, rates of exposure, MDA compliance, and rounds of MDA completed (Helmy ***et al.***, 2006). Secondly, the samples used for the current research were collected in September 2006 and stored at -20^{o}C. Although unlikely, there exists the potential for loss of sample integrity, leading to lower antibody reactivity, with continued freezing and thawing of samples.

To preserve integrity of samples for seroepidemiological studies it is important to use not only the correct collection method, but also correct storage prior to testing. Serum collection in endemic countries is often infeasible, not only for difficulties concerning sample preservation, but also for cumbersome large-scale sampling that is required (Hitch ***et al.***, 1991). Serum collection is predominantly by venepuncture since serum collection via fingerprick is problematic. Lastly, there can also be logistical difficulties for collecting serum in isolated communities. There are many advantages for collecting blood by fingerprick. These include, but are not limited to,

- Lesser side effects for the patient (including reduced risk of haematoma);
- Lesser problem for disposal of sharps;
- Smaller volume of equipment needed;
- Less invasive procedure;
- Reduced risk of needlestick injury to the person collecting the blood; and,

- Reduced risk of blood contact to laboratory scientist.

The reduced risk of needlestick injury is particularly important in areas where night bleeding is still required, or areas where there is potential transmission of blood-borne infectious diseases such as HIV, Hepatitis B and C (Rodriguez-Perez ***et al.***, 1999). It must be emphasised that this research compared two sampling techniques; filter paper vs. plasma/serum and not collection techniques; fingerprick vs. venepuncture, since blood was collected for both samples using the fingerprick method. Using the fingerprick method to collect blood for separation of serum/plasma would not be feasible for epidemiological surveys because of the difficulty of obtaining adequate volumes with this technique and, if using capillary EDTA tubes, the increased cost.

Collecting the blood onto filter paper using the fingerprick method would provide a favourable alternative and has been successful for the Og4C3 antigen assay (Itoh ***et al.***, 1998; Wattal ***et al.***, 2007). It is an easy sample to collect and also can be easily obtained from groups where venous collection is difficult such as children (Terhell ***et al.***, 1996). Another favourable aspect of filter paper sampling is that in other NTD programmes, such as the OCP, the introduction of filter paper techniques has increased the number of volunteers willing to participate in blood collection, again contributing to economic feasibility and ease of surveying (Rodriguez-Perez ***et al.***, 1999).

Altering the sample collection method requires optimisation. OD readings from filter paper samples were observed to be higher than serum, potentially from interference with other blood proteins (Spencer, 1988; Riemekasten ***et al.***, 2002; Dalby ***et al.***, 2009). Therefore, a different negative cut-off value may be required to reflect the higher OD absorbance values. This hypothesis was confirmed as the mean + 3SD of 45 eluates derived from non-endemic individuals was found to be OD ≤ 0.260, higher than the provided negative control. The manufacturer currently recommends a negative cut-off value of OD ≤ 0.200, which is clearly too low for filter paper sampling (Appendix 1). This abolishes the conservative positive cut-off value for serum (OD ≥ 0.250) (Weil ***et al.***, 2010) and confirms the manufacturer's choice for OD ≥ 0.400. Consequently, filter paper sampling in this research abided by a positive cut-off value of OD ≥ 0.400 and a negative cut-off value of OD ≤ 0.260.

The outcome of the comparison of eluates derived from whole blood dried onto filter paper with plasma was promising. The less conservative cut-off of OD ≥ 0.250 was used for the plasma samples, when compared to filter paper sampling, according to previous research (Weil ***et al.***, 2010). Due to isolated field conditions, transportation and storage of serum was impractical in the current study, so plasma samples were used for the comparison. The ELISA specifications for the filter paper eluate were favourable with a high sensitivity and specificity of 92.3% and 76.5% respectively. Disappointingly, a low PPV of 60.0% implied that filter paper sampling may result in false positives at an approximate rate of 40.0% (95%-CI 24.9% to 56.7% of the time). To increase the PPV, by increasing the positive cut-off value

(OD ≥ 0.400), would compromise assay sensitivity. This would be detrimental since high sensitivity would be paramount for surveillance and detecting any potential resurgence early.

The alleged false positivity was not observed in previous controlled laboratory experiments using the Filariasis CELISA (Weil ***et al.***, 2010). Here the researchers utilised blood spiked with a known amount of antibody onto filter paper (Weil ***et al.***, 2010). The reason for the discrepancy in the field setting could be linked to the serum dilution factor. It was assumed that 5 µl of serum was eluted from 10 µl of blood. If the individual was anaemic with a low haematocrit, the serum to whole blood ratio would increase and potentially more than 5 µl could be eluted. Eluting a higher volume of serum from an antibody positive individual would alter the OD readings of the assay.

Testing eluates derived from blank filter paper soaked in diluent did not result in a higher reading than diluent alone (data not shown); thus eliminating the filter paper itself as a source of contamination. Secondly, the high reading was unlikely due to cross-reactivity with other parasites. This is because serum specificity studies were promising (Weil ***et al.***, 2010) and non-endemic individuals also had higher OD absorbance values when assayed for a negative cut-off. This consequently raised the question concerning the difference between blood and plasma samples. The presence of haemoglobin was unlikely to affect absorbance readings since haemoglobin absorbance peaks at a wavelength of 540 nm (Zingraff ***et al.***, 1979) and the

assay itself is read at a dual wavelength of 450 nm and 650 nm. Therefore, further investigation was required to block potential non-specific reactions.

The inherent higher OD absorbance values observed for filter paper sampling were further investigated for the potential of interfering blood proteins and/or enzymes using two methods; blocking buffer and H_2O_2 treatments respectively. It was expected that if either method was necessary, then the OD absorbance values would significantly reduce following treatment. Plates are pre-treated with blocking buffer during the manufacturing process and washing buffers included in the kit contain foetal bovine serum for added effect (Spencer, 1988). Addition of a blocking step containing 5% skim milk and 0.05% Tween-20 was thought to be sufficient if non-specific protein binding was occurring. This was because previous research indicated 0.5% casein and 0.05% Tween-20 was the most effective concentrations for blocking non-specific binding (Kenna ***et al.***, 1985; Vogt ***et al.***, 1987; Spencer, 1988). Secondly, the potential for endogenous peroxidase from blood samples to result in higher OD absorbance values can be reversed through the addition of a 3% H_2O_2 blocking step (De Haas ***et al.***, 2008).

Although addition of a blocking step prior to sample incubation did not significantly alter the OD absorbance values, there was a significant reduction when H_2O_2 treatment was included. This indicated that the higher OD absorbance values may be unaffected by the presence of blood proteins, but could be affected by endogenous peroxidase. During sample preparation, endogenous peroxidase can potentially leak from the red cells,

which has been found to occur with the Og4C3 ELISA, and other ELISAs based on whole blood samples (De Haas ***et al.***, 2008) (Appendix 2). The substrate detection system of the ELISA relies on the reaction of the horseradish peroxidase, linked to the secondary conjugate, with the substrate. If endogenous peroxidase is also present, and not washed off the plate thoroughly, it can potentially react with the secondary conjugate thus reducing the colour reaction. The endogenous peroxidase can be quenched by inclusion of H_2O_2 treatment prior to substrate incubation. Despite the significant reduction in OD absorbance values following H_2O_2 treatment, it was believed that this may not have been due to endogenous peroxidase. This is because the detection system for Filariasis CELISA is TMB substrate (Weil ***et al.***, 2010). TMB substrate contains H_2O_2, which reacts with the horseradish peroxidase to produce the colour reaction measured as OD absorbance. If the external H_2O_2 treatment is not thoroughly washed from the wells, it is conceivable that it would interfere with the detection system altering assay results. Consequently, the ELISA method currently recommended by the manufacturer was considered suitable for filter paper sampling without modifications. However, the higher observed OD absorbance for filter paper sampling requires further investigation, including higher stringency blocking conditions such as salt buffers or higher concentrations of Tween-20, casein, or foetal bovine serum (Spencer, 1988; Jorgensen ***et al.***, 2005).

Forty percent of samples potentially being false positive (a low PPV) may appear disadvantageous. However, from a programmatic perspective, the

low PPV should not impact greatly on survey work since overall increases in antibody prevalence rates would detect a problem, rather than individual results. If follow-up studies in problem areas were required, than serum samples could be used for confirmation if necessary. The high sensitivity and NPV are the crucial aspects for seroepidemiological studies since all positives are likely to be detected with limited false negatives. Consequently, the high NPV and sensitivity observed in this study, when using filter paper sampling, are advantageous for the LF programme. The question of alleged false positivity and slightly higher OD absorbance values for filter paper sampling requires further investigation in terms of the impact on post-MDA prevalence surveys (Chapters 5, 6, and 7).

Variations in secondary conjugate reagent could potentially occur during transit. To investigate this scenario, batches of kits were dispatched, half by road and half by air transport. Positive control titrations resulted in a linear relationship of OD absorbance values. The OD absorbance values clustered around the line of equality illustrating a concordance correlation coefficient of 87.3%. Consequently, exposure of the reagents to ambient temperature for three days did not impair reagent integrity. However, it is advisable to refrigerate kits immediately upon arrival and to transport kits at 4^{o}C, especially when long transit times are expected such as overseas dispatch.

Finally, sample integrity could potentially be affected by storage conditions. The decrease in sensitivity observed following 10 months of storage was in agreement with previous studies, which assayed antibodies against

O. volvulus (Rodriguez-Perez ***et al.***, 1999). Rodriguez-Perez ***et al***. (1999) observed a significant decrease in antibody detection following seven months of filter paper storage at -70^{o}C, -20^{o}C, 4^{o}C and ambient temperature. Therefore, the results from the current study suggest that in order to detect individuals with low antibody titres, filter paper testing should occur within the first 10 months of storage. The filter papers utilised in the previous study were the Whatman No.2 papers, which may be less robust than the Tropbio filter paper discs. This is because the Whatman No.2 papers are more delicate and are not designed to soak a specific volume of blood. To ascertain the effect of storage on blood-soaked Tropbio filter paper discs, further storage studies need to be conducted looking at several time points and storage conditions, including -70^{0}C, -20^{0}C, 4^{o}C and ambient temperature. Since it was not ascertained when during the 10 months storage that reactivity decreased significantly, time points should ideally include three months, six months, and nine months.

For filter paper sampling, the high NPV, coupled with high assay sensitivity, would be advantageous as LF prevalence declines in endemic countries, adding to the usefulness of the assay in post-MDA surveys or future surveillance work. Although filter paper sampling requires overnight elution, the sample preparation is quicker and easier. Filter paper sampling requires submerging the protrusion in sample diluent prior to elution, whereas serum samples are diluted by pipetting. Both serum collection and preparation can be cumbersome when large sampling is involved. As LF prevalence

declines, antibody serology is likely to supersede other LF diagnostic assays in terms of early detection.

In summary, the key findings from the research described in this chapter were:-

1) There was a high specificity recorded for sera samples when using the Filariasis CELISA (94.7%);
2) OD absorbance values steadily decreased as sera with high titres of antibodies were titrated;
3) OD absorbance values for filter paper samples derived from non-endemic individuals showed slightly higher values than the negative control provided with the kit;
4) Filter paper sampling performed comparably well compared to plasma sampling;
5) Delayed transport at ambient temperature did not adversely affect the kit reagents; and,
6) Reactivity of whole blood eluted from filter papers significantly decreased following storage for 10 months at -20^{o}C.

Thus, it can be concluded that:-

1) The Filariasis CELISA has high specificity and sensitivity and is superior to ELISAs based on crude filarial extract;
2) The current recommendation for assaying samples at 1:100 is acceptable;

3) For filter paper sampling, a higher negative cut-off of OD absorbance value ≤ 0.260 is required;
4) The commercial Filariasis CELISA performed comparably well to the research-based Bm14 assay prototype;
5) The filter paper collection technique, using fingerprick blood, for the detection of antibodies, using the Filariasis CELISA, is a feasible and favourable option for future seroepidemiological surveys. It is more cost-effective and less laborious than serum collection; and,
6) The low PPV requires further investigation to ascertain its effect on prevalence rates (Chapters 5, 6 and 7).

CHAPTER 5

POST MASS DRUG ADMINISTRATION SURVEYS IN FOUR SOUTH PACIFIC COUNTRIES: DEFINING CESSATION OF LYMPHATIC FILARIASIS TRANSMISSION

5.1 INTRODUCTION

Countries in the South Pacific are reaching the end of their target MDAs and are now in a position to declare themselves LF-free (Huppatz ***et al.***, 2009). Consequently, countries entering the surveillance mode need to be vigilant to maintain levels of LF prevalence below thresholds required for ongoing transmission. Therefore, an appropriate surveillance strategy for the South Pacific must be implemented. These strategies must include efficient diagnostic tools and achievable surveying methods on an ongoing basis.

Currently, the recommended WHO strategies include stratified sampling or LQAS (WHO, 2000; Huppatz ***et al.***, 2008). Limitations exist for both sampling methods including their effects on under-resourced health systems and inaccuracy of reflecting the heterogeneic nature of LF transmission (Washington ***et al.***, 2004; Gambhir ***et al.***, 2010). LQAS involves sampling at least 3000 schoolchildren, born after the initiation of effective MDAs, and "failing the lot" if one CFA positive child is observed (WHO, 2005). This has been deemed excessive and beyond the scope of what is required for successful elimination (Weil and Ramzy, 2007). Stratified sampling is also

inappropriate for declaring areas LF-free or for future surveillance. This is because key residual areas, also referred to as “hotspots”, can be overlooked. This phenomenon was observed in India where scattered areas had persistent Mf prevalence ≥ 1% in the population for 20 years (Ramaiah ***et al.***, 2009). The authors from the latter study concluded that current surveying and diagnostic methods are inadequate in this stage of the LF elimination programme. This highlights the urgent need to evaluate the current WHO guidelines.

Targeting a specific cohort of children using antibody testing would alleviate these issues and also allow implementation of surveillance surveys to be coupled with other school based health programmes (Massara ***et al.***, 2006; Hotez ***et al.***, 2007; Enk ***et al.***, 2008; Gyapong ***et al.***, 2010). This is to promote future incorporation of surveys into primary care health systems to ensure their ongoing implementation and sustainability (Hopkins, 2009; Gyapong ***et al.***, 2010). Integrated surveys have the potential to reduce costs and field work (Sturrock ***et al.***, 2009). This has been demonstrated in southern Sudan, whereby a combined survey for LF and loiasis was conducted by one paid group of health workers, rather than sending separate teams (Sturrock ***et al.***, 2009). Reducing costs is especially important as international bodies reduce funding because programme activities must be sustainable by the respective country’s health budget (Gyapong ***et al.***, 2010).

Recently, CTS have been proposed as a potential method for surveillance mode for the Pacific as part of the LF surveillance strategy for the PICT

(WHO, 2007a; Huppatz, 2008). This method relies on detecting CFA positive children in either school based or community based surveys. The strategy was based on the pre-approved LF diagnostic assays available and took into consideration resource and funding constraints (Huppatz, 2008). Upon detection of a CFA positive child, using the field ICT, surrounding residents (200 m radius or 24 houses) would be tested to identify the Mf positive index case from the child's house of residence (Huppatz, 2008). However, as the prevalence of LF declines, the sensitivities of the gold standards, which measure Mf and CFA, also decrease (Chanteau ***et al.***, 1994; Rocha ***et al.***, 1996; Grady ***et al.***, 2007; Njenga ***et al.***, 2007a; Molyneux, 2009), potentially making these assays less useful in this stage of the LF elimination programme (Durrheim ***et al.***, 2003; Melrose ***et al.***, 2004; Washington ***et al.***, 2004).

There may be many months of exposure to infective mosquitoes before infection occurs (Hairston and de Meillon, 1968; Southgate, 1984; Ichimori and Crump, 2005) and that person becomes CFA positive, and another lag phase before that person becomes Mf positive, (Ramzy, 2002; Durrheim ***et al.***, 2003; Rawlins ***et al.***, 2004; Grady ***et al.***, 2007; Weil and Ramzy, 2007). It has also been demonstrated, in a cohort of Haitian children, that although filarial infection could be acquired early in life, it required months before the intensity of the infection increased to detectable CFA levels (Lammie ***et al.***, 1998). In low prevalence settings, the limitations of the aforementioned diagnostic assays would be detrimental to the LF programme. This is because resurgence would not be detected early and, if so, transmission

would intensify to levels requiring rigorous control efforts. This problem could be solved by implementing the Filariasis CELISA to detect antibodies in children because it will allow earlier detection during stages of parasite exposure.

Since drafting the surveillance strategy (Huppatz, 2008), four South Pacific countries have finished their CTS either alone or in conjunction with post-MDA prevalence surveys (Huppatz ***et al.***, 2009) and it is the aim of this chapter to draw conclusions on the appropriateness of using this strategy for ongoing surveillance or the potential for including antibody serology. The current study focussed on the CTS in Tonga, Tuvalu, Vanuatu and Samoa.

Surveys differed among countries by measuring either one or a combination of diagnostic parameters. These were Mf, CFA, and/or antibody prevalence in children ≤ 10 years. The first two parameters measure infection status, whereas antibody serology is an indicator of continuing exposure to infection (Dissanayake and Ismail, 1987; Weil ***et al.***, 1997). Post-MDA surveys conducted across the Pacific were designed to demonstrate cessation of LF transmission and thus the potential to move from annual MDAs to surveillance mode (WHO, 2007a). It is critical to confidently define this interruption of transmission since LF resurgence in the South Pacific has been previously reported for French Polynesia (Chanteau ***et al.***, 1995; Esterre ***et al.***, 2001; Burkot ***et al.***, 2002; Huppatz ***et al.***, 2008), Samoa (Kimura ***et al.***, 1985) and the Cook Islands (PacELF, 2006). Thus, the diagnostic method chosen must allow swift and accurate surveillance to allow

effective control of the resurgent areas to minimise impact and quickly reduce transmission below the threshold.

Antibody responses to LF exposure have been suggested as an ideal immunological measurement for identifying areas of residual endemnicity in future surveillance campaigns (Lammie ***et al.***, 2004; Washington ***et al.***, 2004; Ramzy ***et al.***, 2006; Grady ***et al.***, 2007; Njenga ***et al.***, 2007a). Previous studies demonstrated that antibody production in response to LF exposure occurs during the first few years of life (Gao ***et al.***, 1994) and, therefore, if cessation of LF transmission has occurred, then children should be antibody negative (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). One standardised diagnostic tool for the detection of antibodies is the Filariasis CELISA. However, the efficacy of the commercial assay in large-scale field sampling is yet to be ascertained.

One important aspect of large-scale field sampling, to be representative of LF transmission across a country, is the choice of areas for study. Previous research has highlighted the shortcomings of using sentinel sites for the post-MDA surveys, as representative of the country's prevalence, since higher awareness of LF and concentrated programmatic activity could contribute to greater success in these areas (Grady ***et al.***, 2007; Mladonicky ***et al.***, 2009). Post-MDA surveys conducted across the Pacific were by stratified cluster sampling design (Huppatz ***et al.***, 2009). Baseline studies at the commencement of PacELF, based on convenience sampling, showed

markedly different LF prevalence rates across the Pacific and the history of the LF programme in each of the four countries chosen for the study differed (PacELF, 2006; Huppatz ***et al.***, 2009).

Filariasis prevalence in Tonga was recorded as early as 1785 (Iyengar, 1965) and the first MDA occurred in 1977 (PacELF, 2006). Following the MDA, Mf prevalence declined from 17.6% to 1%, and by 1999 when Tonga joined PacELF the CFA prevalence was recorded as 2.7% (PacELF, 2006). Annual MDA rounds were completed in Tonga for five years from 2001 to 2005 with reported coverage rates of 79%, 84%, 91%, 86% and 85% respectively (PacELF, 2005; Huppatz ***et al.***, 2009). Coverage rates would never reach 100% since children ≤ 2 years, pregnant women, and individuals with serious pre-existing health conditions such as cancer are excluded (PacELF, 2006). Coverage rates use population census as the denominator (WHO, 2005). Now that the final MDA has been completed, Tonga has reached the target CFA prevalence of < 1% of the population (0.4%) following the post-MDA prevalence survey in 2007 (Huppatz ***et al.***, 2009), which is PacELF's recommended threshold for interrupting LF transmission (PacELF, 2006). Following the post-MDA survey, the CTS was conducted to include ICT testing in children aged between 5 and 6 years as well as antibody serology, the results of which are presented in this chapter.

The small coral atoll of Funafuti, the main island of Tuvalu, recorded a high CFA baseline level at the beginning of the PacELF in 1999 of 22.3% (PacELF, 2006). Since then, five rounds of MDA were completed from 2001

to 2005 with coverage rates reported for the first four years of 71%, 47%, 82%, and 83% respectively (PacELF, 2006). A national survey conducted in 2004 found that the CFA prevalence had reduced by 50% to 11.9% of the whole population (PacELF, 2006). The final MDA has been completed (2005) and the results of the CTS and antibody serology are presented in this chapter.

Prior to PacELF, a baseline survey carried out in Vanuatu in 1998 found the country Mf prevalence to be 2.5% and the CFA prevalence to be 4.8% (PacELF, 2006). By 2005 Vanuatu had completed its fifth and final round of MDA with annual rounds from 2000 to 2004 with reported coverage rates of 83%, 84%, 84%, 87% and 85% respectively (Huppatz ***et al.***, 2009). Following the final round of MDA in 2005, the follow-up post-MDA prevalence survey showed a countrywide CFA prevalence of 0.2% with no detectable Mf positives (Huppatz ***et al.***, 2009). Following this survey, the CTS was conducted to include ICT testing in children aged between 5 and 6 years as well as antibody serology, the results of which are presented in this chapter.

Samoa has a long history of filariasis control; initial filariasis surveys began as early as the 1920s, with attempts at control programmes in the 1940s (Ichimori and Crump, 2005). In 1966 MDAs began, and Samoa completed 10 rounds of MDA before the establishment of PacELF (Burkot ***et al.***, 2002; Ichimori ***et al.***, 2007b). In 1999, Samoa was the first country to implement the MDA regime under the direction of PacELF (Ichimori and Crump, 2005) and a further seven rounds of MDA were completed from 1999 to 2008. The

reported MDA coverage for the five rounds conducted from 1999 to 2003 was 90%, 57%, 68%, 60% and 80% respectively (PacELF, 2005; Huppatz ***et al.***, 2009). A stratified cluster nationwide survey of Samoa in 2003, carried out following the five rounds of MDA, demonstrated an overall Mf prevalence of 0.4% with a CFA prevalence of 1.1% (Huppatz ***et al.***, 2009). This corresponded to a 75.6% reduction in CFA positive individuals since the implementation of the PacELF (Ichimori and Crump, 2005; Ichimori ***et al.***, 2007a). The promising decline in CFA prevalence led to a sixth round of MDA in 2006 with the goal of further lowering the prevalence below the recommended threshold of 1% antigenaemia (PacELF, 2006). The results from the follow-up survey in 2007 are presented in conjunction with the country's CTS.

It was the hypothesis of this research that the addition of antibody serology would either complement or supersede the need for CFA testing. Consequently, the purpose of this research was to assess the potential for antibody serology to identify hotspots. It was also the purpose to assess the feasibility of CTS and/or antibody serology for defining cessation of transmission.

5.2 AIMS

The specific aims for the work described in this chapter are to:-

(1) Assess the efficacy of the Filariasis CELISA in a field setting with large sampling numbers;

(2) Assess the suitability of antibody serology for defining endpoints and future surveillance of LF;

(3) Assess the current CTS for its feasibility for defining a cessation of LF transmission; and,

(4) Assess serological antibody mapping as a tool to identify potential hot spots of residual endemnicity.

5.3 MATERIALS AND METHODS

5.3.1 Study Population

Four Pacific countries; Samoa, Tonga, Tuvalu, and Vanuatu, participated in the study as part of their required post-MDA CTS (Figure 3.1). Overall, LF prevalence was calculated for the four countries by using SPSS Version 17.0 and 95%-CI were calculated and included in the figures (Section 3.3). Prevalence rates were compared using Chi-squared analysis.

5.3.1.1 Samoa

The post-MDA 2007 survey was a randomised national survey, by health district, across both Savai'i and Upolu using the stratified cluster method. Stratified cluster sampling was performed by dividing the country into IUs within which villages were chosen randomly (WHO, 2005). Within the village, a minimum of five households were chosen randomly. Residents of the chosen households were all encouraged to participate. Overall, the study included 6648 people, with 2315 children ≤ 10 years. Of these children, 1045 were also tested for antibodies. These children were aged 5 to 10 years.

Participants of any age were asked to register and bloods were taken. All participants were bled for ICT testing (Section 3.2.5.1) and those that tested ICT positive were re-bled for Mf testing (Section 3.2.4). Filter paper samples were also collected from children ≤ 10 years for antibody serology, as measured with the Filariasis CELISA (Section 3.2.3.1 and Section 3.2.6).

5.3.1.2 Tonga

CTS carried out in Tonga was in accordance with the LF surveillance strategy for the PICT (Huppatz, 2008), but with the addition of antibody serology. Blood collection occurred in primary schools across the islands of 'Eua, Ha'apai and Vava'u. Overall, 790 children aged 5 or 6 years participated in the study (Figure 5.1). All participants were bled for ICT

testing (Section 3.2.5.1) and those that tested ICT positive were re-bled for Mf testing (Section 3.2.4). Filter paper samples were also collected for antibody serology using the Filariasis CELISA (Section 3.2.3.1 and Section 3.2.6).

Figure 5.1: Post-MDA survey in Tonga. Children aged 5 and 6 years attending primary school across three island groups of Tonga were asked to participate. Bloods were taken for ICT and antibody serology.

5.3.1.3 Tuvalu

Two hundred and twenty one children aged ≤ 10 years were chosen, using convenience sampling, from primary schools located on the main island of Funafuti during a cross-sectional survey. All participants were bled for ICT

testing (Section 3.2.5.1) and those that tested ICT positive were re-bled for Mf testing (Section 3.2.4). Filter paper samples were also collected for antibody serology using the Filariasis CELISA (Section 3.2.3.1 and Section 3.2.6).

5.3.1.4 Vanuatu

A total of 3840 children aged 5 or 6 years were bled across the country in accordance with the CTS outlined in the LF surveillance strategy for the PICT (Huppatz, 2008), but with the addition of antibody serology. These children were bled for ICT (Section 3.2.5.1) and those positive were bled for Mf testing (Section 3.2.4). Filter paper samples were also collected for antibody serology using the Filariasis CELISA (Section 3.2.3.1 and Section 3.2.6).

5.3.2 Statistical Analysis and Seroprevalence Mapping

All data was entered into SPSS Version 17.0. Prevalence rates were calculated using the descriptive options in SPSS and 95%-CI were calculated (Section 3.3). For Samoa, the prevalence rates among the three parameters of Mf, CFA, and antibodies were investigated using scatter plots and Spearman's correlation coefficient. Chi-squared test was used to investigate the difference in prevalence rates within countries as data was recorded as categorical (positive/negative).

Prevalence of antibody positivity across the four countries was mapped using ArcGIS 9.3 (ESRI, 2008) and referred to as serological (seroprevalence) mapping. For Samoa, the mapping also included Mf and CFA prevalence of total population screened.

5.4 RESULTS

5.4.1 Study Population

Overall prevalence rates are depicted in Figure 5.2. Mf and CFA prevalence rates for Samoa included adults and children. In Vanuatu, all children tested negative for CFA. Similarly, all children in Tonga were CFA negative. This was coupled with a significantly lower antibody prevalence recorded for both Vanuatu (6.0%) and Tonga (6.3%) ($P < 0.001$) when compared to Tuvalu and Samoa (Figure 5.2). In Samoa, there were children positive for both Mf and CFA (Figure 5.3) and this was complemented with a significantly higher antibody prevalence (30.7%) ($P < 0.001$) than the other countries (Figure 5.2). CFA positive children (4.1%) were detected in Tuvalu, which recorded an intermediate antibody prevalence (Figure 5.2).

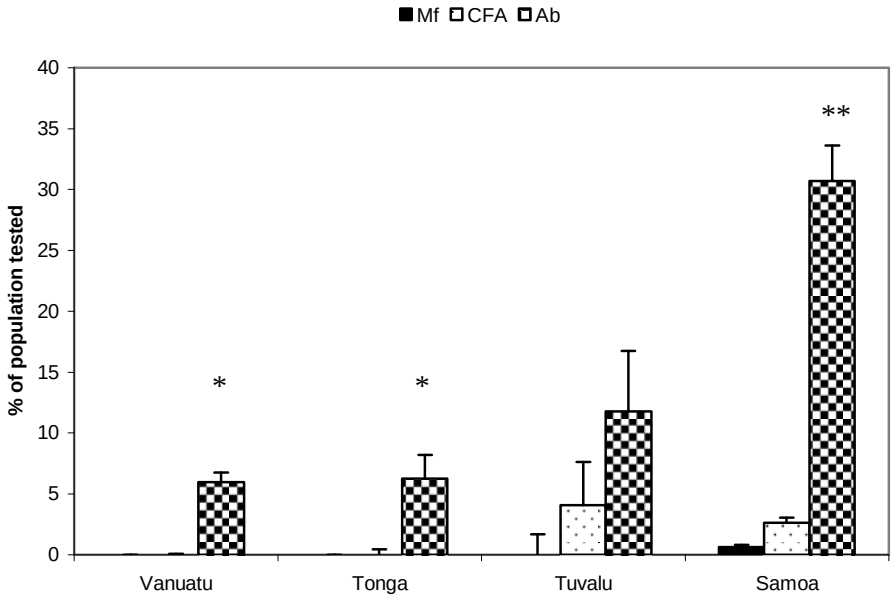


Figure 5.2: Prevalence rates of the diagnostic parameters: Mf, CFA and antibody (Ab). Mf and CFA prevalence for Samoa included adults and children. CFA positive children were detected in Tuvalu and Samoa. There were also Mf positive children in Samoa (Figure 5.3). There was a significantly lower antibody prevalence in Vanuatu and Tonga (*) and a significantly higher antibody prevalence in Samoa (**) ($\chi^2 = 549.3$; df = 3; $P < 0.001$). There were no Mf or CFA positive children detected in either Vanuatu or Tonga.

5.4.1.1 Samoa

Overall, antibody prevalence recorded in children assayed was 30.7% (Figure 5.2). Mf positive children were recorded in the 2007 survey (Figure 5.3). When comparing the two islands of Samoa, Upolu had a significantly higher proportion of Mf and CFA positives ($P = 0.002$ and $P < 0.001$ respectively) than Savai'i, although no differences were observed between the antibody prevalence in children (Table 5.1). When analysing the data by health district, Leulumoega 1, on Upolu, had a significantly higher Mf prevalence (2%) ($P < 0.001$) and CFA prevalence (7.3%) ($P < 0.001$)

(Figure 5.4). Coupled with this, and the results for Palalui, Leulumoega 1 had a significantly higher antibody prevalence in children (44.9%) ($P < 0.001$) (Figure 5.4). Prevalence for the three parameters was easily visualised on the geographical maps, including potential problem areas in the aforementioned districts (Figure 5.5).

When analysing the data for children only, Upolu maintained a significantly higher CFA prevalence ($P = 0.043$), yet no differences were observed for Mf prevalence between the islands ($P = 0.218$) (Figure 5.6). For the health districts, Mf positive children were found only in Leulumoega 1, which had a significantly higher antibody prevalence (Figure 5.4), and the highest CFA prevalence, although not significantly higher than the other villages with CFA positive children ($P = 0.271$). Lufilufi and Palalui were the other two villages with CFA positive children (Figure 5.6).

The relationship between Mf prevalence, CFA prevalence and antibody prevalence was also investigated (Figures 5.8, 5.9, 5.10). It was found that there was a significant correlation between the prevalence of Mf and CFA (Figure 5.7; $P = 0.016$), but not CFA prevalence and antibody prevalence in children (Figure 5.8; $P = 0.432$). There was not an observed linear correlation between Mf prevalence and antibodies in children (Figure 5.9; $P = 0.939$).

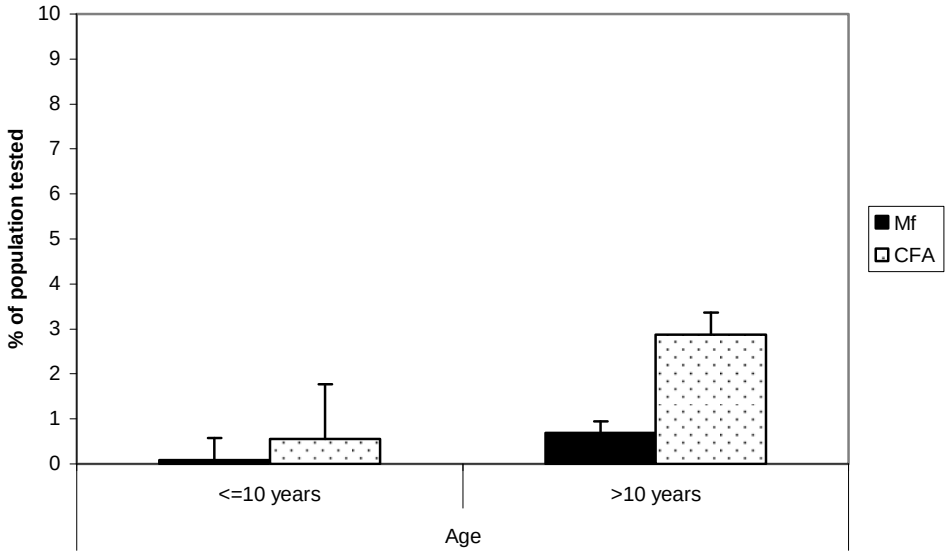


Figure 5.3: Mf and CFA prevalence in children ≤ 10 years and the rest of the population tested as part of the post-MDA survey in 2007 in Samoa. Mf and CFA positives were detected in children aged ≤ 10 years in the post-MDA survey, which was conducted in seven health districts across Savai'i and Upolu (Figure 5.4).

Table 5.1: Data collected from the post-MDA 2007 national survey in Samoa. Upolu had a significantly higher Mf and CFA prevalence than Savai'i ((χ^2 = 9.6; df = 1; P = 0.002) and (χ^2 = 27.2; df = 1; P < 0.001) respectively). No differences were observed for antibody prevalence (χ^2 = 0.638; df = 1; P = 0.424).

Island	***Mf (%)***	***CFA (%)***	***Antibody in children (%)***
Savai'i	**0.25** (7/2738) (95%-CI 0.1-0.5)	**1.4** (39/2738) (95%-CI 1.0-1.9)	**29.6** (163/550) (95%-CI 25.9-33.7)
Upolu	**0.86** (32/3710) (95%-CI 0.6-1.2)	**3.5** (131/3710) (95%-CI 3.0-4.2)	**31.9** (158/495) (95%-CI 27.8-36.2)

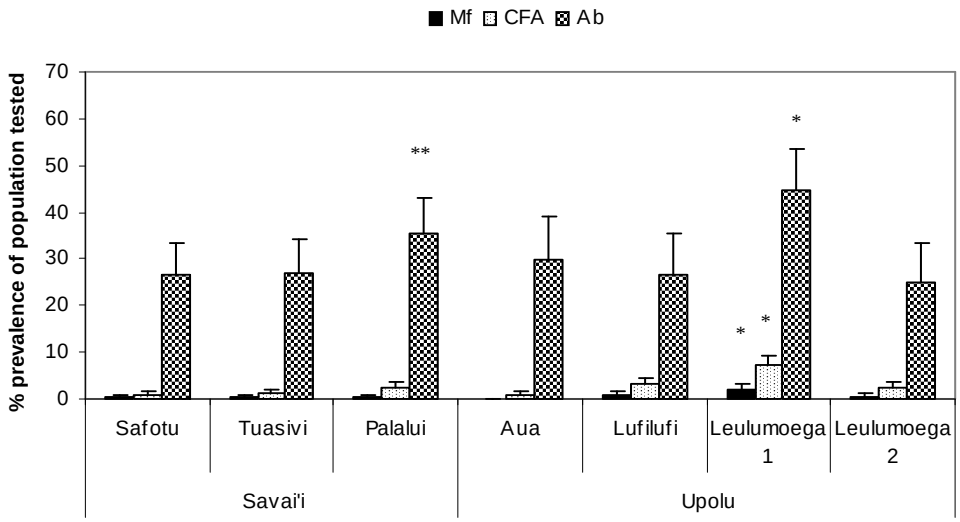


Figure 5.4: Mf, CFA and antibody (Ab) prevalence across the health districts in Samoa. Leulumoega 1 recorded a significantly higher Mf (*), CFA (*) and antibody (Ab) prevalence (*) than the other districts (χ^2 = 46.9; df = 6; P < 0.001) (χ^2 = 124.9; df = 6; P < 0.001) (χ^2 = 20.6; df = 6; P < 0.001). Similarly, Palalui recorded a significantly high antibody prevalence (**) in children (χ^2 = 20.6; df = 6; P < 0.001).

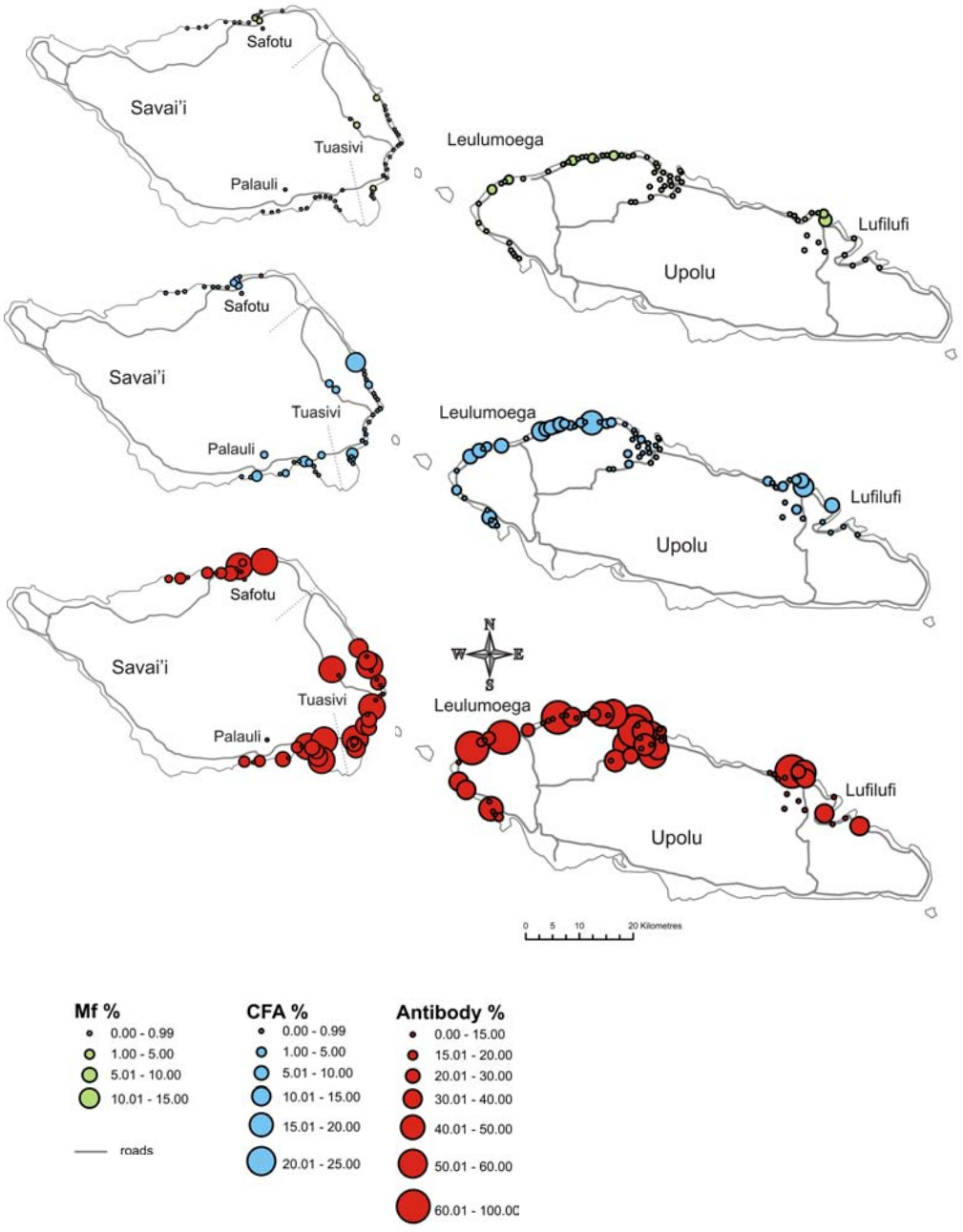


Figure 5.5: Serological mapping for Mf, CFA and antibody prevalence across Samoa. Geographical mapping allows quick reference to suspected problem areas or "hot spots", such as seen in Leulumoega on the island of Upolu

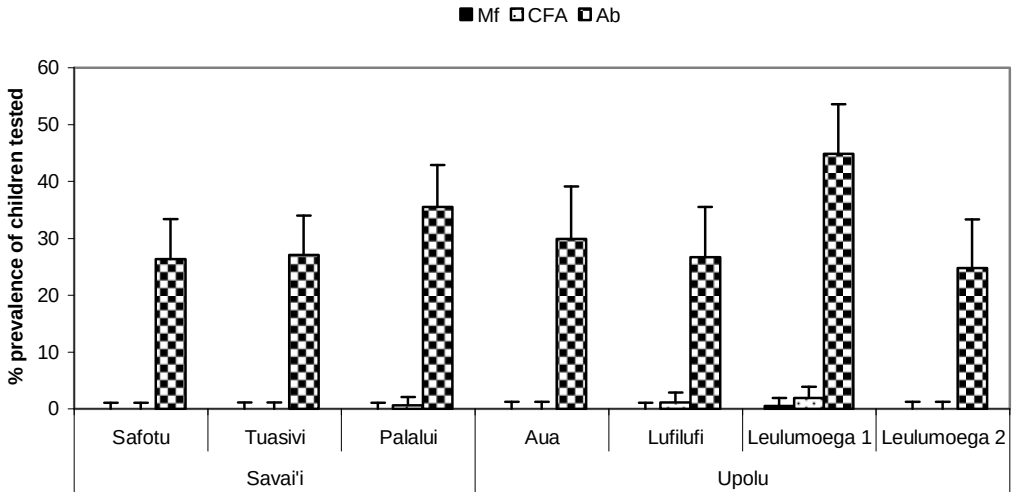


Figure 5.6: Mf, CFA and antibody (Ab) prevalence in children ≤ 10 years only across the health districts in Samoa. Similar to the data across all ages groups, Upolu had a significantly CFA prevalence in children than Savai'i (χ^2 = 4.086; df = 1; P = 0.043). No differences for Mf prevalence were observed between the two island groups (χ^2 = 1.514; df = 1; P = 0.218). Similarly, no significant differences for Mf prevalence were observed among the health districts (χ^2 = 10.694; df = 6; P = 0.098), since Mf positive children were identified only in Leulumoega 1. Complementing this result, Leulumoega 1 recorded a significantly higher antibody prevalence than the other districts (χ^2 = 20.6; df = 6; P < 0.001). The health districts of Aua, Leulumoega 2, Safotu and Tuasivi recorded no CFA positive children. There were no significant differences for CFA prevalence in children among the health districts (χ^2 = 2.612; df = 2; P = 0.271).

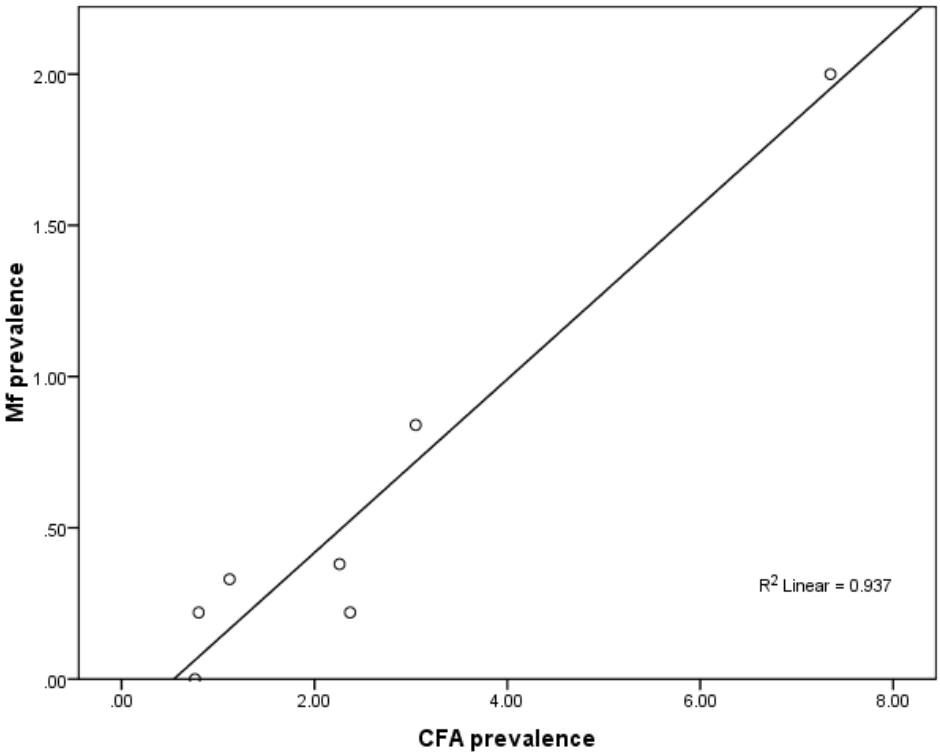


Figure 5.7: Correlation between the prevalence of Mf and CFA positive adults and children in Samoa. There was a significant linear correlation between Mf and CFA prevalence in adults and children in Samoa (r_s = 0.85, P = 0.016).

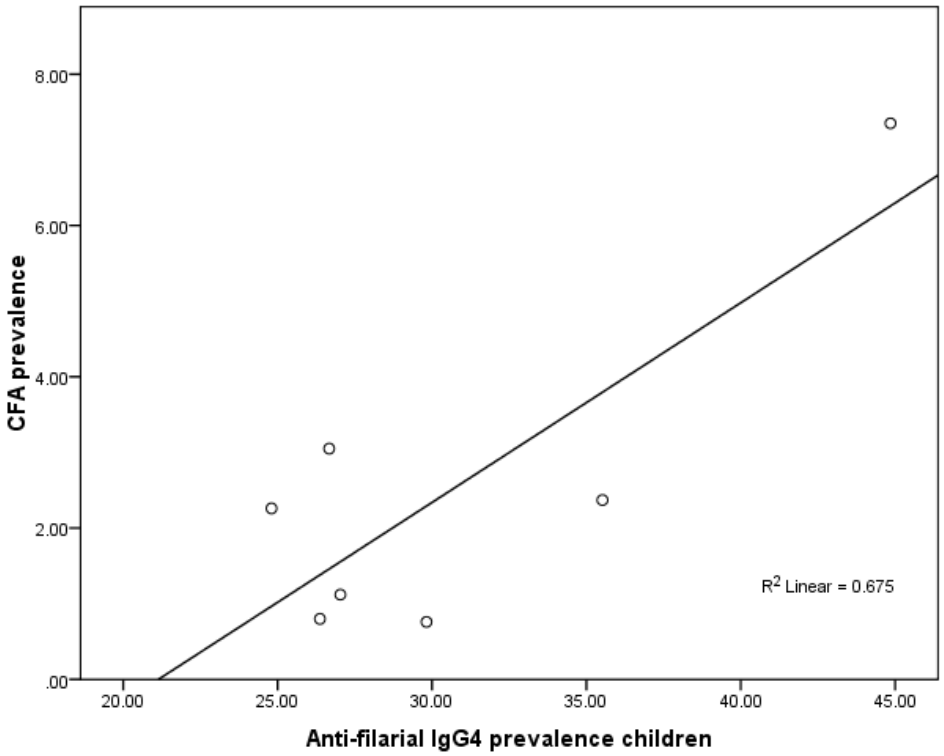


Figure 5.8: Correlation between CFA prevalence and antibody prevalence in children in Samoa. Although a linear relationship between CFA and antibody prevalence in children in Samoa was observed, this was not significant (r_s = 0.36; P = 0.432).

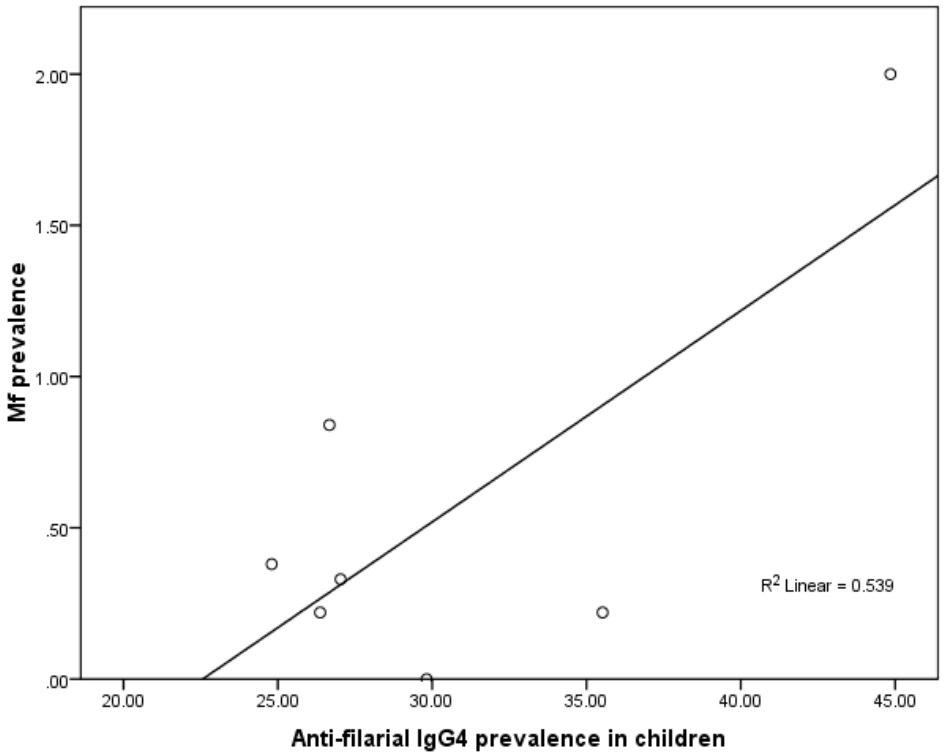


Figure 5.9: Correlation between Mf prevalence and antibody prevalence in children in Samoa. There was no significant relationship observed between the prevalence of Mf and antibody prevalence in children (r_s = 0.4; P = 0.939).

5.4.1.2 Tonga

Overall, antibody prevalence recorded for children was 6.3% (Figure 5.2). When comparing the three island groups; 'Eua, Ha'apai, and Vava'u, no significant differences for antibody prevalence were found (Figure 5.10; P = 0.067).

The seroprevalence map for the three island groups identified two possible "hotspots" where antibody prevalence in children exceeded 20% (Figure 5.11). However, the sample size in these villages was relatively

small, and the identification of one or two positive children increased the ratio of positivity.

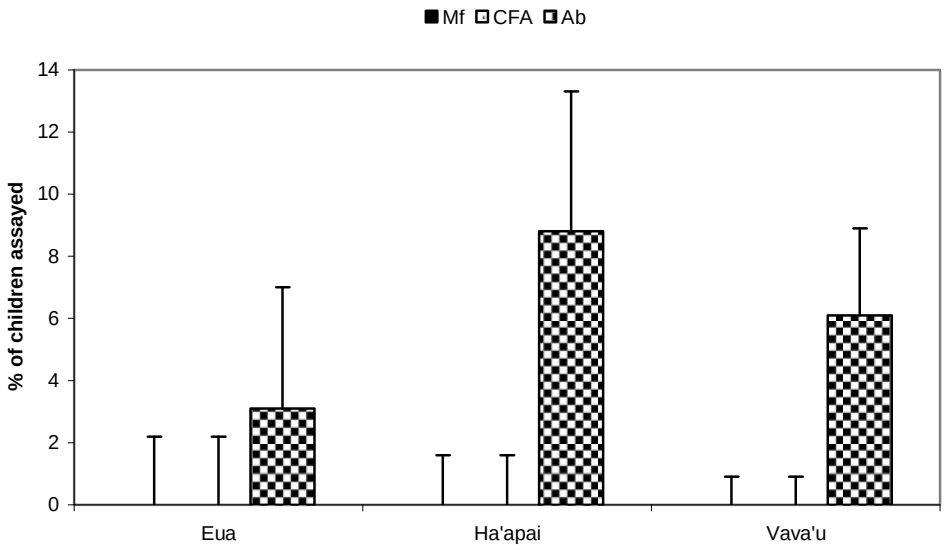


Figure 5.10: Mf, CFA and antibody (Ab) prevalence rates in children residing in Tonga. No Mf or CFA positive children were detected. Antibody prevalence rates in children did not significantly differ among the island groups ($\chi^2 = 5.4$; df = 2; P = 0.067).

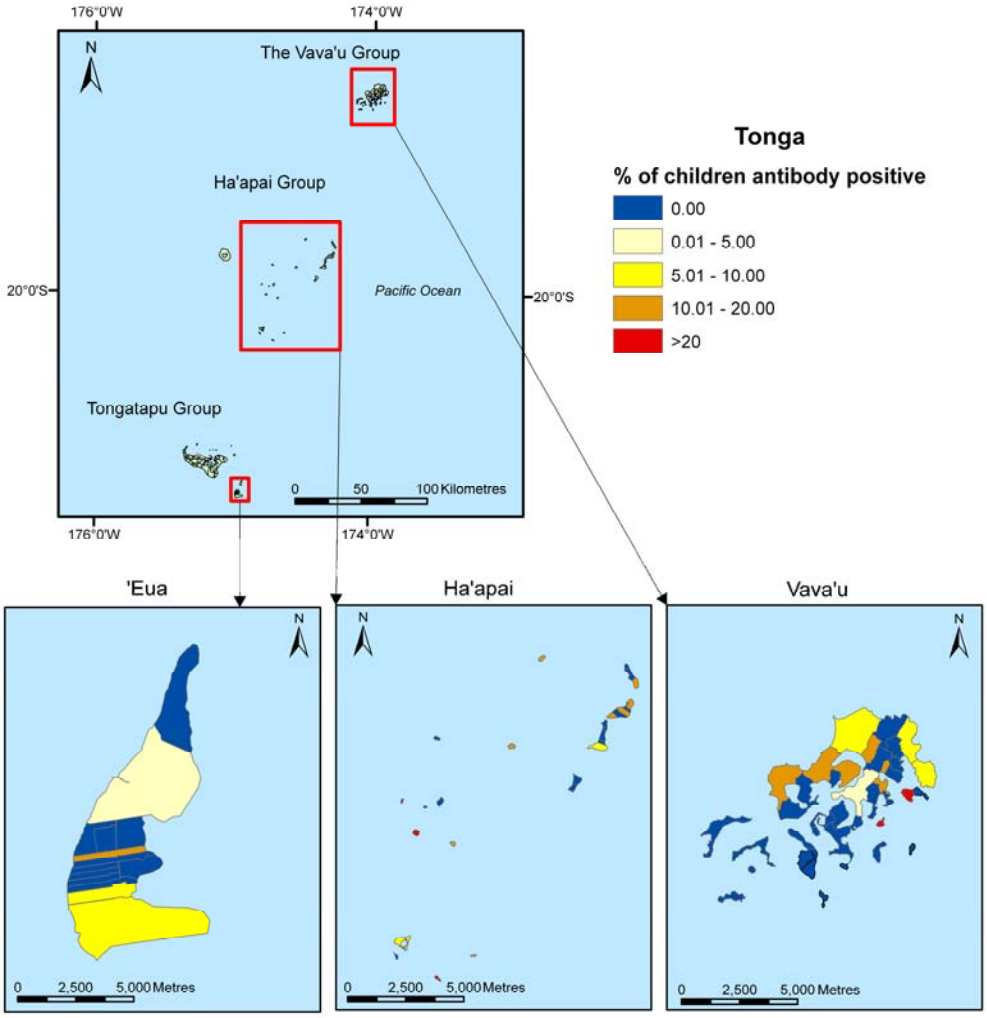


Figure 5.11: Seroprevalence map: antibody prevalence of children residing in villages in Tonga. Although no CFA positive children were detected, observation of the seroprevalence map revealed two villages in Vava'u (Koloa and Olo'ua) where > 20% of children tested were antibody positive. However, total numbers of children tested per village were relatively small, resulting in a high proportion of positives.

5.4.1.3 Tuvalu

Of the 221 children surveyed, nine CFA positives were detected, giving CFA prevalence in children of 4.1% (Figure 5.2). The antibody prevalence recorded for Tuvalu was 11.8% (Figure 5.2).

Only the main island of Funafuti was investigated, which is divided into eight villages. Of the eight villages, it was observed that Naumasa (45.5%) and Teone (41.7%) had significantly higher antibody prevalence rates than the rest of Funafuti (Figure 5.12; $P < 0.001$). Similarly, the village of Naumasa had a significantly higher CFA prevalence in children than Alapi and Senala ($P = 0.023$), but not Lofeagai or Tekavatoetoe ($P = 0.773$).

The higher CFA and antibody prevalence recorded for Naumasa was easily observed using serological mapping (Figure 5.13).

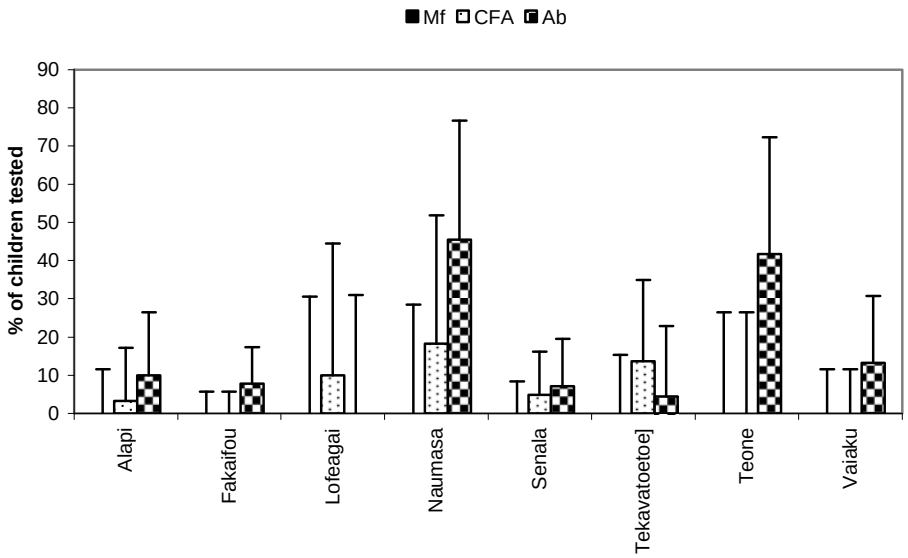


Figure 5.12: Mf, CFA and antibody (Ab) prevalence rates in children residing in Tuvalu. No Mf positive children were detected. Naumasa and Teone had a significantly higher antibody prevalence than the other villages ($\chi^2 = 26.7$; df = 7; $P < 0.001$). Naumasa had a significantly higher CFA prevalence ($\chi^2 = 16.2$; df = 7; $P = 0.023$).

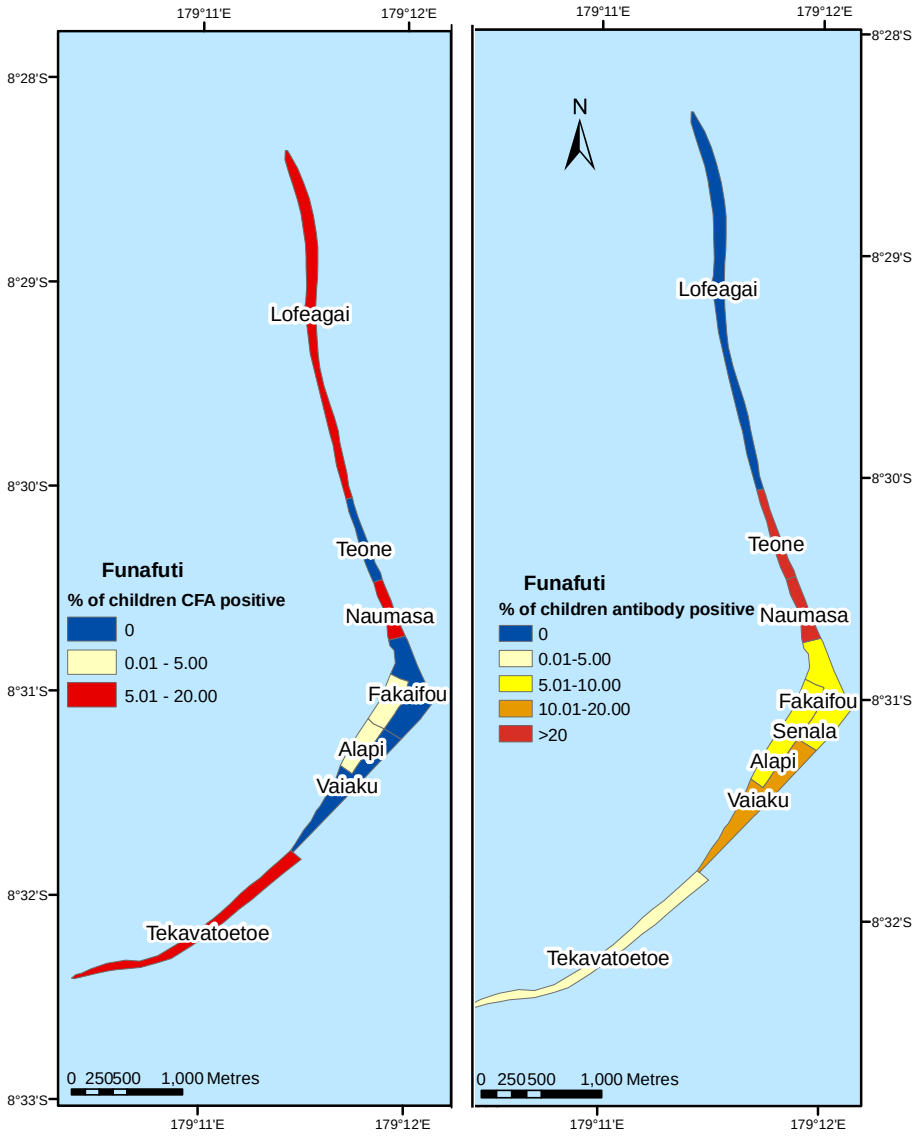


Figure 5.13: Serological mapping for CFA and antibody prevalence in children residing in villages in Tuvalu. The highest antibody prevalence was recorded for the neighbouring villages of Teone and Naumasa, the latter corresponding to a high CFA prevalence. High CFA prevalence was also observed for both ends of the island of Funafuti.

5.4.1.4 Vanuatu

Statistical analysis within the country of Vanuatu was unable to be performed accurately. This was because children registered as part of the survey were recorded in two different ways. Some children were registered by their school of attendance; thus making it difficult to trace back to their village of residence which could be on a different island. Other children were registered by their village of residence. This made it difficult to statistically compare the antibody prevalence between island groups.

This also affected the accuracy of the seroprevalence mapping since prevalence was mapped according to the database (Figure 5.14). That is, some children were mapped as part of the community survey and others as part of the school based survey. Given the nature of the sample collection, seroprevalence mapping was still included to show the importance of each country choosing only one method for sample collection. Secondly, if there was identification of problem areas, these areas could be scrutinised by the LF programme manager to ascertain if children were surveyed by school or community.

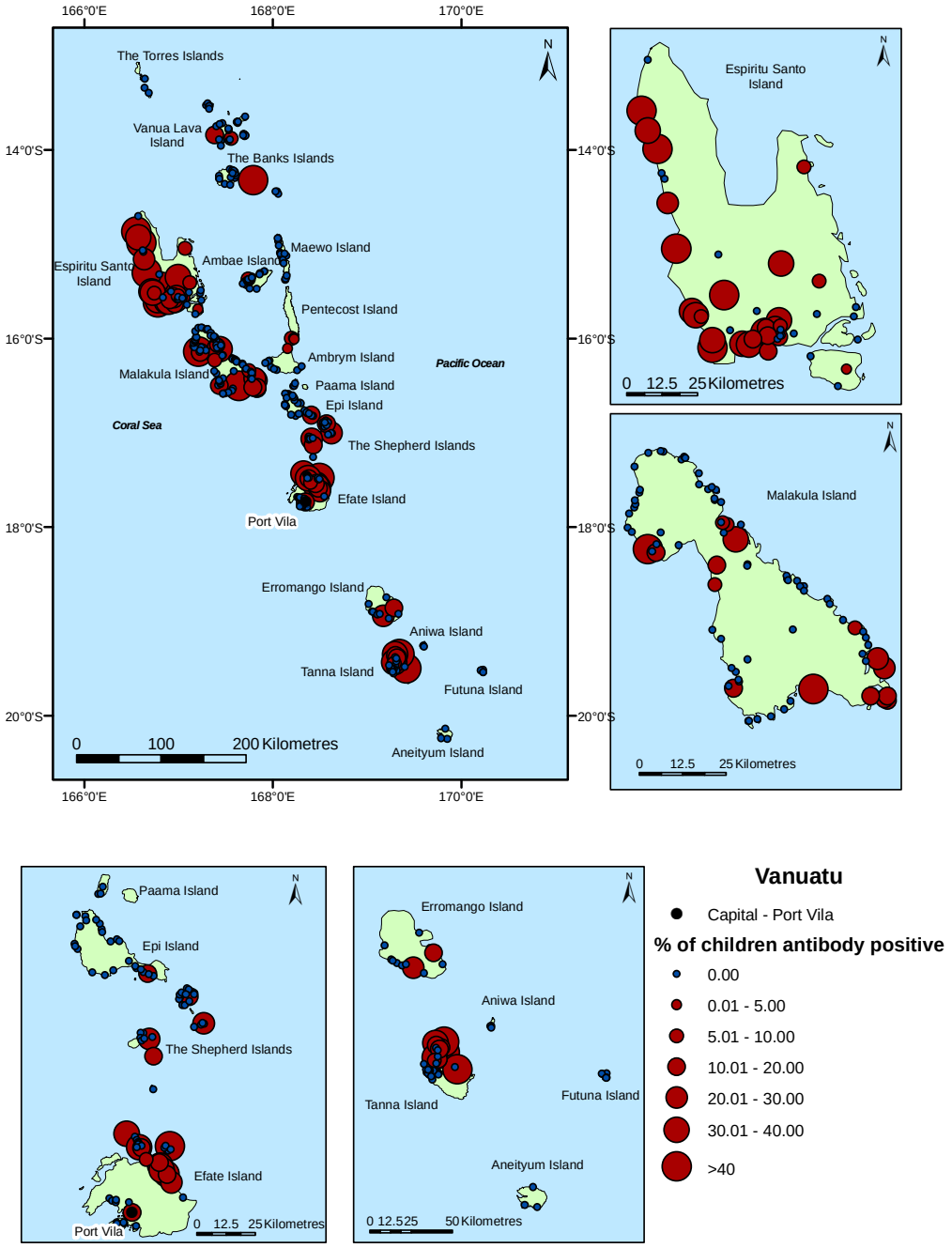


Figure 5.14: Seroprevalence map: antibody prevalence of children residing in villages in Vanuatu. Although no CFA positive children were detected, observation of the seroprevalence map revealed a number of villages where > 20% of children tested were antibody positive. However, total numbers of children tested per village were relatively small, resulting in a high proportion of positives.

5.5 DISCUSSION

Inclusion of antibody serology, assayed by the Filariasis CELISA, would be advantageous to the LF programme. The assay was easily adaptable for large scale field sampling and antibody serology identified key residual areas of endemnicity in Samoa which would otherwise have been overlooked. This is crucial information for country programme managers.

It was hypothesised that Tonga and Vanuatu would have a low antibody prevalence since in 2006 Tonga recorded an overall CFA prevalence of 0.4% and Vanuatu recorded an overall CFA prevalence of 0.2% in 2005 (Huppatz ***et al.***, 2009). These prevalence rates were well below the PacELF recommended target of < 1% CFA prevalence definition for cessation of LF transmission (PacELF, 2006). It would be expected that the persistence of low antibody titres would result in some reactive samples. Samoa continued to have a CFA prevalence > 1% in 2004 (Huppatz ***et al.***, 2009); therefore, it was hypothesised that antibody prevalence in children would be high. Lastly, Tuvalu reported a CFA prevalence of 12.1% following a survey of the whole population in 2004 (PacELF, 2006). It was hypothesised that Tuvalu would also have high antibody prevalence in children. These hypotheses were supported, demonstrating the value of the Filariasis CELISA for detecting antibodies in a large sampling cohort with filter paper sampling.

The currently proposed CTS did appear to reflect a cessation of LF transmission in both Tonga and Vanuatu since no CFA positive children were

identified. This was complemented by a significantly lower antibody prevalence for both Vanuatu and Tonga when compared to Tuvalu and Samoa. Identification of Mf and CFA positive children in Samoa was coupled with a significantly higher antibody prevalence in children than for the other countries. This could be indicative of ongoing transmission of LF. Tuvalu recorded intermediate antibody prevalence, and 4.1% of children were CFA positive, again suggesting ongoing transmission of LF. The usefulness of the CTS for detecting residual areas of endemnicity was further explored within each country.

The current surveillance strategy for the Pacific, using CFA testing in children (CTS), was not adequate for identifying key residual areas of endemnicity in Samoa. In Samoa, CFA positive children were found in Lufilufi, Palalui and Leulumoega 1, the latter also having Mf positive children. Coupled to these findings, a significantly higher antibody prevalence was also recorded for Palalui and Leulumoega 1. Antibody prevalence exceeded 10% in all health districts, possibly because Mf positive adults were detected in all health districts except for Aua. Aua recorded CFA prevalence below the < 1% recommended threshold (PacELF, 2006). Collectively, those health districts containing Mf positive adults, but no CFA positive children, would not be detected as problem areas by analysis of the CTS alone. Thus, the current strategy is lacking the sensitivity required to identify hotspots. Clearly, inclusion of antibody serology would solve this problem. In-depth epidemiological studies need to be conducted to further investigate the usefulness of incorporating antibody serology, assayed by the Filariasis

CELISA, using the health districts of Lufilufi, Palalui and Leulumoega 1 (Chapter 6).

Antibody prevalence in children was a reflection of the CFA prevalence in the community and, therefore, an indication of ongoing transmission. In Samoa, there was a significant correlation between the prevalence of Mf and CFA, concurring with previous studies in high prevalence areas (Lammie ***et al.***, 1994; Sunish ***et al.***, 2001; Sunish ***et al.***, 2002; Ramzy ***et al.***, 2006; Malla ***et al.***, 2007). Although for Samoa the linear relationship between CFA prevalence and antibody prevalence in children did not reach significance, the village with the highest CFA prevalence also had a significantly high antibody prevalence in children. Similarly, children residing in the village of Naumasa in Tuvalu had a significantly higher CFA prevalence and antibody prevalence than children from the other villages. Collectively, antibody serology reflected prevalence of infection in Samoa and Tuvalu.

It has been strongly suggested that the current WHO guidelines necessitating continuing MDA rounds if one in 3000 children born after the MDA is positive for CFA (WHO, 2005) are excessive and beyond what is needed for successful LF elimination (Weil and Ramzy, 2007). The suggested target, based on studies in Egypt where the vector ***Culex*** sp. is endemic (Weil ***et al.***, 1999), was a < 2% antibody prevalence in first-year primary school children (Weil and Ramzy, 2007). The data presented here does not concur with this suggested definition since 6% and 6.3% of first-year primary school children in Vanuatu and Tonga respectively were antibody positive and no Mf or CFA

positive children were recorded. However, the researchers did conclude that different threshold targets may be required for different endemic areas based on the variation in vector species, baseline LF prevalence, transmission patterns, biting rates, and the MDA regime implemented (Weil and Ramzy, 2007). The preliminary data presented in the current study suggests that South Pacific countries require a higher antibody threshold target, but this would require further investigation in other low prevalence settings. Importantly, the inherent higher OD absorbance values (Chapter 4) did not falsely represent ongoing LF transmission since seroprevalence was low in those countries where < 1% CFA prevalence had been achieved (Huppatz ***et al.***, 2009).

In Samoa, the significantly higher antibody prevalence in children, with Mf and CFA positives in children, is indicative of continuing LF transmission. These findings from the 2007 survey support previous research, where Samoa had the highest post-MDA survey results compared to four other Pacific countries, which had also completed the obligatory five rounds of MDA (Huppatz ***et al.***, 2009). Unfortunately, ***Ae. polynesiensis***, the major vector for LF transmission in Samoa, has been shown to increase transmission efficacy as the prevalence of LF declines (Bryan and Southgate, 1976; Samarawickrema ***et al.***, 1985b; Burkot ***et al.***, 2006; Ichimori ***et al.***, 2007b). Secondly, the lack of MDA compliance has been identified as a factor which impedes successful elimination (Kyelem ***et al.***, 2008). Consequently, countries with persistent transmission, despite MDA intervention, should be the focus of community surveys to ascertain levels of

compliance (Chapter 8) (Burkot ***et al.***, 2002; Babu and Kar, 2004; Mathieu ***et al.***, 2006; Gunawardena ***et al.***, 2007; Huppatz ***et al.***, 2009). Even in India, persistent antigenaemia has been recorded in an endemic area which has undergone nine rounds of MDA (Mukhopadhyay, 2010). The information gained from the serological maps in the current study would provide the Samoan LF programme managers with a starting point to identify problem areas, based on high exposure (antibody positivity) in children. The reasons for ongoing transmission in Samoa are yet to be ascertained. It is crucial that this is addressed in order for successful LF elimination. Inclusion of vector control programmes may be necessary for achieving success as well as directly observed therapy (DOT), which is lacking in the Pacific (Burkot ***et al.***, 2002; Huppatz ***et al.***, 2009).

Serological antibody mapping (seroprevalence mapping) would be advantageous during LF surveillance and identifying areas of residual endemnicity. It allows visualisation of problem areas where exposure occurs (Booth and Dunne, 2004). Despite a countrywide low level antibody prevalence in Tonga and Vanuatu, upon mapping there was marked geographical variation. This may highlight geographical regions that could be potential areas of concern in the future. Problem foci could be clearly identified in Tuvalu, where the highest levels of exposure were identified in Naumasa (45.5%) and Teone (41.7%) which, upon further statistical analysis, were significantly higher than the other Tuvaluan villages. In Tonga, the two identified problem villages where seroprevalence was > 20% require further investigation for Mf and CFA prevalence as well as administration of DEC.

This is also true for those villages in Vanuatu. In Samoa, mapping Mf, CFA and antibody prevalence provided an excellent way to identify “hotspots” which were further investigated (Chapter 6).

Seroprevalence surveying is advantageous compared to parasitological surveys alone in that seroprevalence is less susceptible to the fluctuations observed with infection prevalence rates that are reliant on seasonality (Booth and Dunne, 2004). It has been shown in malaria that the infection prevalence varied dramatically depending on the mosquito population in the wet and dry season whereas exposure patterns (antibody titres) remained constant (Booth and Dunne, 2004). Consequently, as a vector-borne disease, LF programmes would benefit from seroprevalence mapping. However, limitations do exist if the dataset is lacking. Villages in Tonga and Vanuatu that were identified as potential hotspots only had a small dataset and one positive child could lead to a high positive ratio. Secondly, Tuvalu recorded a high CFA prevalence in children, yet only 221 children were registered in the survey. Thus, seroprevalence mapping must be viewed with caution if using a small dataset. Secondly, visualising problem areas would only be useful for the LF programme if exposure related geographically to Mf/CFA positive individuals responsible for ongoing transmission. This requires further investigation (Chapter 7).

There are few, if any, shortcomings from the present study. These shortcomings relate to constraints on health workers, including the need to oversee many health programmes (Hopkins, 2009). Consequently, there

were issues with data management since the survey conducted in Vanuatu registered children by two different methods. Secondly, in remote areas, villages can often be known by two different names. These issues are easily corrected by having a standard surveillance strategy.

In summary, the key findings from the research described in this chapter were:-

1) Cessation of LF transmission in Tonga and Vanuatu was associated with no detectable CFA positive children and significantly low levels of antibody prevalence;
2) Ongoing transmission of LF in Samoa and Tuvalu was associated with CFA positive children and, in Samoa, Mf positive children. Both countries had a high prevalence of antibody positive children;
3) Most importantly, in Samoa, testing children for CFA positivity alone would have missed important residual positive foci, as defined by Mf prevalence exceeding 1%; and,
4) Seroprevalence mapping resulted in an easy visualisation of areas of residual endemnicity, which require further investigation (Chapter 6).

Thus it can be concluded that:-

1) The current surveillance strategy for the Pacific should be complemented with antibody serology;

2) Antibody serology in cohorts of children, as measured by the Filariasis CELISA, indicated the level of LF transmission; and,
3) The Filariasis CELISA would be a beneficial addition to the current repertoire of LF diagnostic assays.

CHAPTER 6

EVALUATION OF CONTINUING TRANSMISSION OF LYMPHATIC FILARIASIS IN SAMOA USING THE FILARIASIS CELISA

6.1 INTRODUCTION

The addition of antibody serology as a complementary test in a low prevalence setting, for either demonstrating cessation of transmission or future surveillance, could counteract the need for large sampling numbers and provide an earlier warning system for potential resurgence (Ramzy, 2002; Durrheim ***et al.***, 2003; Rawlins ***et al.***, 2004; Grady ***et al.***, 2007; Weil and Ramzy, 2007). However, the relationships between Mf, CFA, and antibody prevalence need to be thoroughly investigated in low prevalence settings before any programmatic decisions are made (Lammie ***et al.***, 2004; Grady ***et al.***, 2007). In addition, previous serological studies have not incorporated the commercial Filariasis CELISA (Lammie ***et al.***, 1998; Weil ***et al.***, 1999; Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Mladonicky ***et al.***, 2009). It is a necessity for the diagnostic assays utilised by the LF programme to be standardised to allow inter- and intra-country comparisons (WHO, 2005). Before inclusion of antibody serology in the WHO protocols can occur, the Filariasis CELISA needs to be studied in an epidemiological field setting to ascertain its usefulness and relationship to the other measured parameters.

Inclusion of antibody prevalence in field studies, using the research-based Bm14 assay prototype, has shown promising results (Helmy ***et al.***, 2006; Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). Conclusions from these studies indicated the usefulness of monitoring antibody levels in children as a possible indication of interruption of LF transmission. Children were highlighted as the ideal sampling population, if they were born after the effective MDA they should have no prior exposure and should test antibody negative (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009).

In Samoa, the post-MDA 2007 survey indicated that Mf, CFA and antibody prevalence in children, as measured by the Filariasis CELISA, was correlated (Chapter 5). This required further investigation using a comprehensive epidemiological assessment. This is imperative if the Filariasis CELISA is to be incorporated in future surveys. Therefore, the aim of the research described in this chapter was to assess the potential for antibody prevalence measured in children born after commencement of MDA to identify persistent areas of residual endemnicity in Samoa and its relationship to Mf and CFA.

6.2 AIMS

The specific aims for the work described in this chapter are to:-

(1) Ascertain how the Filariasis CELISA complements already approved diagnostic assays in the field setting and how these results compare to previous studies with the research-based Bm14 assay prototype; and,

(2) Investigate correlations of Mf, CFA and antibodies, with age and gender.

6.3 MATERIALS AND METHODS

6.3.1 Study Area

This study was conducted in May 2008, prior to the seventh MDA round in June 2008, on both islands of Samoa (Figure 6.1). Study areas chosen on the island of Savai'i were Tafua and Puapua. Study areas chosen on the island of Upolu included Fasitoo-Tai, Siufaga and Falefa. These five villages have been used as sentinel sites not more than twice since 1999 and were selected to give a range of infection prevalences based on the previous C survey completed in 2007 (Chapter 5) (Table 6.1). Siufaga was chosen as being representative of a LF-free village, as it was previously thought that LF transmission had been interrupted since CFA prevalence was recorded as < 1% in previous years (WHO Samoa, ***personal communication by Dr Kevin Palmer***).

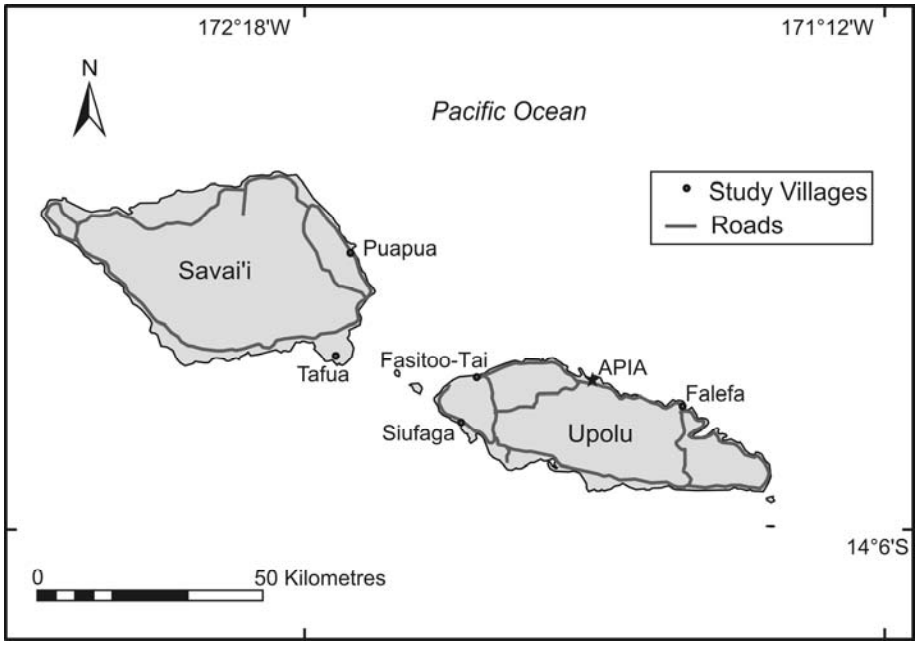


Figure 6.1: Location of the five study villages in Samoa. On Savai'i the two villages were Tafua and Puapua. On Upolu the three villages chosen were Fasitoo-Tai, Siufaga and Falefa. The capital city, Apia, is included on the map as a reference.

Table 6.1: Data collected from the post-MDA 2007 national survey in Samoa. The villages chosen for the research described in the current chapter were based on these 2007 data, and methodology was previously described in detail (Chapter 5). Briefly, Mf and CFA results included the entire screened population. The antibody results were collected from children aged ≤ 10 years.

Island	***Health District***	***Village***	***Mf (%)***	***CFA (%)***	***Antibody (%)***
Savai'i	Palalui	Tafua n = 92	0.5	14.8	71
	Tuasivi	Puapua n = 29	0.2	16.7	40
Upolu	Lufilufi	Falefa n = 122	0.5	10.7	44
	Leulumoega 1	Fasitoo-Tai n = 65	0.6	21.5	25
	Leulumoega 2	Siufaga†	0	0	0

n = number of participants
† = Siufaga was chosen as a negative control village (ceased transmission)

6.3.2 Study Population

It was the aim of the research to screen every individual residing in the villages of Tafua, Puapua and Siufaga ≥ 2 years old, and coverage rates achieved ranged from 79% to 84% of the population (Table 6.2), with the denominator based on the most recent population census (2006). The villages of Fasitoo-Tai and Falefa had populations exceeding 1000 and it was the aim of the study to screen a minimum of 500 residents. The selection criteria for the latter villages related to the previous 2007 survey (Table 6.1). An individual from each village, who tested CFA positive in the previous 2007 survey, was randomly selected. Their household of residence was deemed the central point and, radiating out, every household was included in the survey until approximately 500 individuals were registered and screened. This method was chosen to be similar to the close contact testing strategy of the LF surveillance strategy for the PICT (Huppatz, 2008). Since surveying occurred during the daytime, school children registered in the study by their guardians, after visiting their household of residence, were tested at their respective primary schools.

Table 6.2: Demographics of the individuals residing in the five villages chosen for the study.

	Upolu			Savai'i	
Characteristic	**Fasitoo-Tai**	**Falefa**	**Siufaga**	**Puapua**	**Tafua**
Male > 10 years	232	197	190	162	131
Female > 10 years	227	206	174	160	127
Male child ≤ 10 years	84	89	80	67	47
Female child ≤ 10 years	74	78	51	59	39
Total tested	**617**	**570**	**495**	**448**	**344**
Population census (2006)	1393	1388	629	552	408
% population screened*	44%	41%	79%	81.1%	84%
Median age, years	19	18	23	18	20
Age range, years	2-90	2-86	2-92	2-85	2-84

*based on population census 2006. Note for Fasitoo-Tai and Falefa it was the aim of the study to test a minimum of 500 individuals radially from a central house

In this research, any statement regarding “children” will refer to participants ≤ 10 years. The reasoning for choosing a target population of ≤ 10 years was due to the timing of the initial MDA. MDAs, under the guidance of the WHO, began in Samoa in 1999 (Ichimori and Crump, 2005) and targeting children born after the initial MDA placed their age at approximately 9 years at the time of the study. Unfortunately, in most situations it was apparent that dates of birth were not recorded for children, thus the selection was based on grade level for children who attended school. Children aged 9 or 10 years corresponded to grade five: thus any child equivalent to grade five was included in the study.

Informed consent was given verbally and individuals were registered for the study with a unique identification number linked to the household of residence. Demographic information was recorded including age and gender. Participants were bled for CFA (Section 3.2.2 and Section 3.2.5) and, if positive, Mf (Section 3.2.4) (Figure 6.2). In the village of Puapua, blood for antibody testing was drawn only from children, whereas antibody

testing was done on every participating individual in the other four villages (Section 3.2.3.1 and Section 3.2.6). The initial protocol was to collect filter papers for children and ICT positives only. However, due to problems in communication, after the village of Puapua it was decided to collect filter papers from all registered participants. This alteration worked in our favour since it allowed us to investigate the relationship between acquisition of antibodies and age.

6.3.3 Statistical Analysis

All data was entered into SPSS Version 17.0. Prevalence rates were calculated, including 95%-CI (Section 3.3). The three analyses used were the Chi-square, scatter plots with Pearson's correlation coefficient, and the Mann-Whitney ***U*** non-parametric analysis.

Figure 6.2: Blood was collected by fingerprick following registration of participants.

6.4 RESULTS

6.4.1 Prevalence

The overall prevalence of Mf, CFA and antibody for the five villages is tabulated (Table 6.3). To account for the possibility of including antibody positive children born prior to the 1999 MDA, data was re-analysed for children ≤ 9 years (Table 6.3). No significant difference was observed between the two antibody prevalence rates (P = 0.917). Mf prevalence was significantly higher in Fasitoo-Tai than the four other villages (χ^2 = 14.7; df = 2; P = 0.001). Fasitoo-Tai also had a significantly higher CFA prevalence (χ^2 = 98.1; df = 4; P < 0.001), total antibody prevalence

(χ^2 = 162.6; df = 3; P < 0.001), and antibody prevalence in children (χ^2 = 125.9; df = 4; P < 0.001) (Table 6.3). Although Fasitoo-Tai had a significantly higher number of Mf cases than Tafua (n = 20 cf n = 2), there was not a significant difference between the two villages for the Mf load in carriers (Z = 0.916; P = 0.36) (data not shown).

The lowest CFA prevalence rates were recorded for Puapua and Siufaga, both of which were significantly lower than the other three villages (χ^2 = 98.1; df = 4; P < 0.001) (Table 6.3). Puapua had lower antibody prevalence in children than all other villages, significantly lower than Fasitoo-Tai, Falefa and Siufaga (χ^2 = 87.7; df = 2; P < 0.001). Except for Siufaga, CFA positive children were observed in four of the villages (Table 6.3), all of which exceeded the threshold of 0.1% antigenaemia (WHO, 2005; PacELF, 2006). All five villages exceeded a CFA prevalence of 1%.

Mf positive persons were identified in both Tafua and Fasitoo-Tai (Table 6.3) and prevalence appeared to increase with age (Figures 6.3b and 6.3e), but this did not reach significance (P = 0.968). CFA prevalence increased significantly with age for all villages (P < 0.001) as did the total antibody prevalence (P < 0.001) (Figure 6.3).

Prevalence of Mf, CFA, and antibodies was higher among males for each village (Figure 6.4). The Mf prevalence among males in Tafua and Fasitoo-Tai (1.1% and 4.7% respectively) was higher than females (0% and 1.7% respectively) albeit not significant (χ^2 = 0.368; df = 1; P = 0.544).

Despite a higher Mf load observed for males, there was no significant difference between males (436 Mf/mL blood) and females (100 Mf/mL) ($Z = 1.29$; $P = 0.195$) (data not shown). CFA was significantly higher in males than females for Tafua (14.1% cf. 2.4% ($\chi^2 = 17.1$; $df = 2$; $P < 0.001$)), Falefa (8.4% cf. 1.8% ($\chi^2 = 13.0$; $df = 1$; $P < 0.001$)), and Fasitoo-Tai (17.7% cf. 11.3% ($\chi^2 = 7.02$; $df = 2$; $P = 0.03$). Although higher in males for Puapua and Siufaga, the difference did not reach statistical significance (($\chi^2 = 2.1$; $df = 1$; $P = 0.147$) and ($\chi^2 = 5.27$; $df = 2$; $P = 0.074$) respectively). Total antibodies were significantly higher in the male population for the four villages studied ($\chi^2 = 50.5$; $df = 2$; $P < 0.001$). Male children had greater antibody prevalence, but this was only significant for Fasitoo-Tai ($\chi^2 = 6.816$; $df = 2$; $P = 0.033$).

The relationship between ICT testing and the Filariasis CELISA was also investigated (Figures 6.5a and 6.5b). Although a linear correlation was observed between CFA and antibody prevalence, both in children and the total population, this did not reach significance ($P = 0.391$ and $P = 0.391$ respectively). Circulating Mf was only detected in Tafua and Fasitoo-Tai; therefore no statistical analyses were performed.

Of those individuals CFA positive, the majority were antibody positive (92.6% (95%-CI 87.5 – 96.2)), which was significantly different to the CFA negative group (57.0% (95%-CI 54.8 – 59.2)) ($\chi^2 = 79.4$; $df = 1$; $P < 0.001$). Analysis of OD absorbance values, which were assumed to indicate antibody titre (Dylewski ***et al.***, 1984), revealed some interesting observations. Mf positive

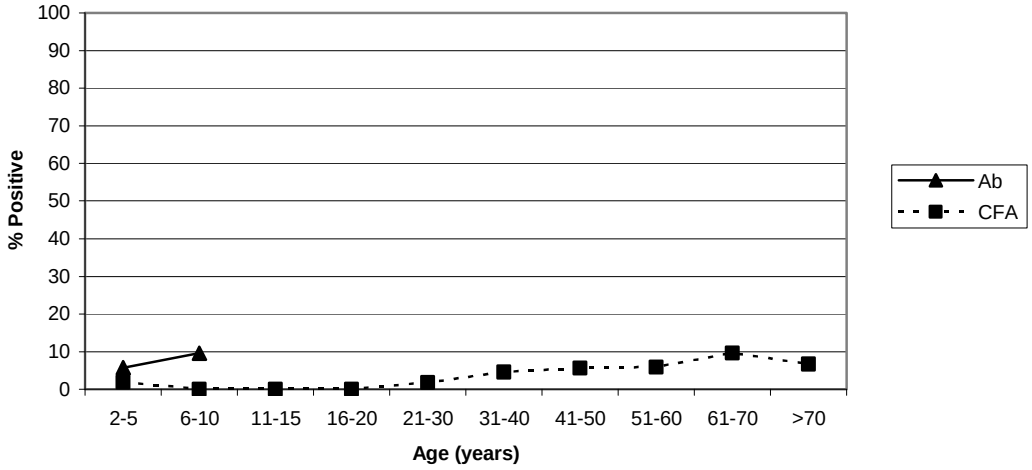
individuals had a significantly higher mean OD absorbance value than the amicrofilaraemic individuals (Figure 6.6; P = 0.038). CFA negative individuals had a significantly lower mean OD absorbance value than CFA positive individuals (Figure 6.6; P < 0.001).

Table 6.3: Prevalence of Mf, CFA and antibodies (Ab) in each of the five villages (%) including 95%-CI. Antibody prevalence was re-calculated to include only children ≤ 9 years to account for the potential of inclusion of antibody positive children born prior to the 1999 MDA. There was no significant difference between the two prevalence rates (Z = 0.104; P = 0.917).

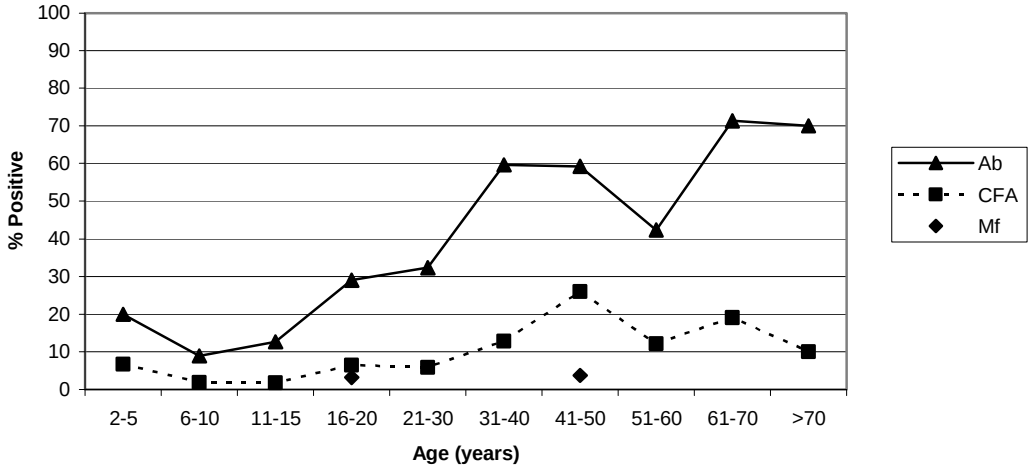
	Upolu			**Savai'i**	
	Fasitoo-Tai	**Falefa**	**Siufaga**	**Puapua**	**Tafua**
Mf prevalence (%)	**3.2** (2.0 – 5.0)	**0**† (0 – 0.7)	**0**† (0 – 0.7)	**0**† (0 – 0.8)	**0.6** (0.1 – 2.1)
CFA prevalence (%)	**14.6** (11.9 – 17.6)	**5.1** (3.4 – 7.2)	**1.6** (0.7 – 3.2)	**2.5** (1.2 – 4.4)	**8.4** (5.7 – 11.9)
Total Ab prevalence (%)	**74.9** (71.3 – 78.3)	**64.9** (60.8 – 68.8)	**64.8** (60.5 – 69.1)	•**ND**	**34.3** (29.3 – 39.6)
Ab prevalence children (%)	**62** (54.0 – 69.6)	**51.5** (43.6 – 59.3)	**46.6** (37.8 – 55.5)	**7.9** (3.9 – 14.1)	**12.8** (6.6 – 21.7)
Ab prevalence ≤ 9 years (%)	**63.1** (54.2 – 71.4)	**49.3** (41.0 – 57.7)	**45.1** (36.1 – 54.4)	**8.9** (4.3 – 15.7)	**14.3** (7.4 – 24.1)
CFA prevalence children (%)	**9.5** (5.4 – 15.2)	**4.2** (1.7 – 8.5)	**0** (0 – 2.8)	**0.8** (0.2 – 4.3)	**3.5** (0.7 – 9.9)

† = Although 0% Mf prevalence was recorded, Mf testing was only performed on CFA positive individuals and not the entire population
•ND = not done

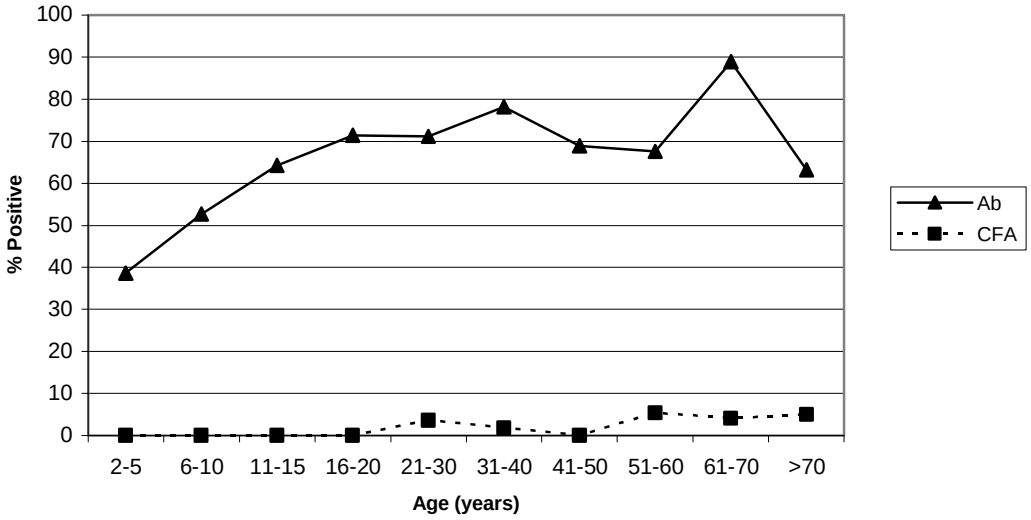
A) Puapua



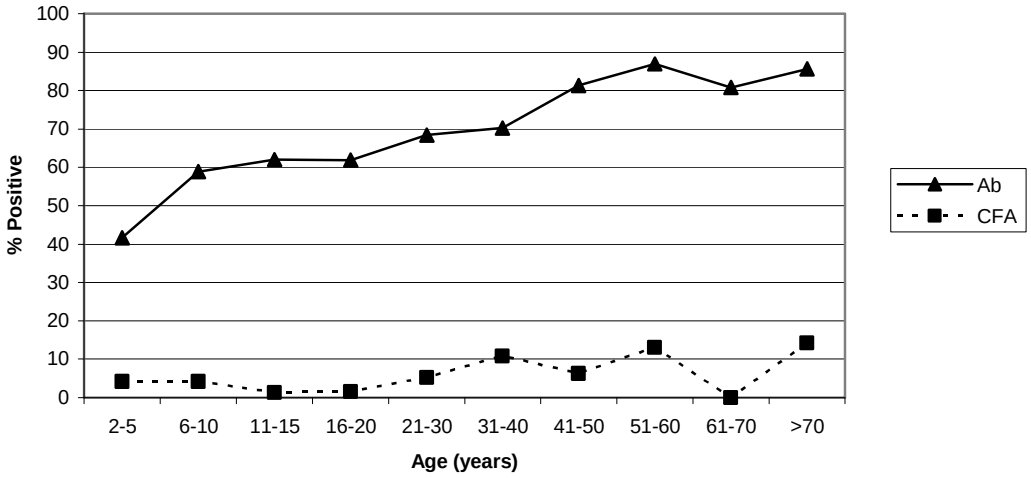
B) Tafua



C) Siufaga



D) Falefa



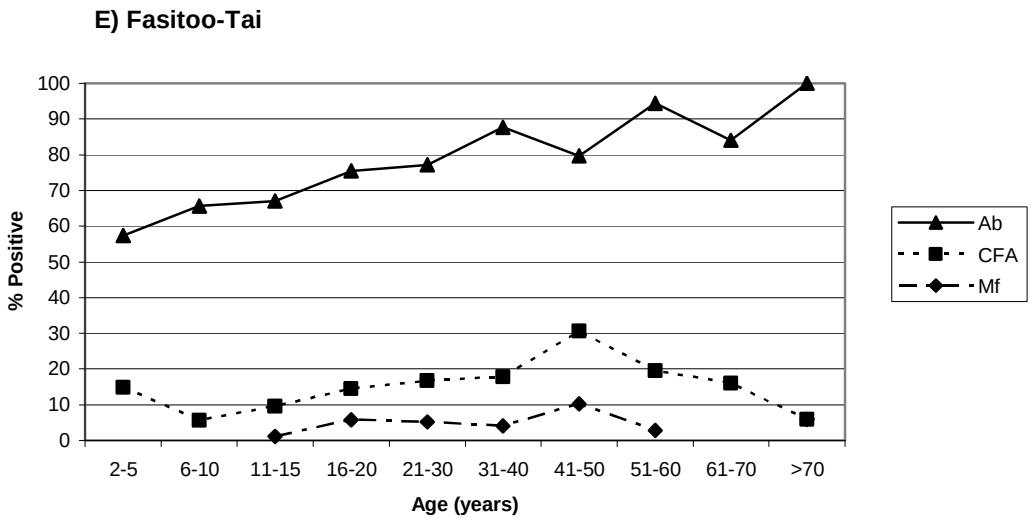


Figure 6.3: Age specific prevalence of Mf, CFA and total antibody (Ab) for each of the five villages A) Puapua, B) Tafua, C) Siufaga, D) Falefa and E) Fasitoo-Tai. CFA and antibody positivity increased significantly with age ((Z = 0.54; P < 0.001) and (Z = 14.2; P < 0.001) respectively). Although it was observed that Mf prevalence also increased with age, this did not reach statistical significance (Z = 0.04; P = 0.968).

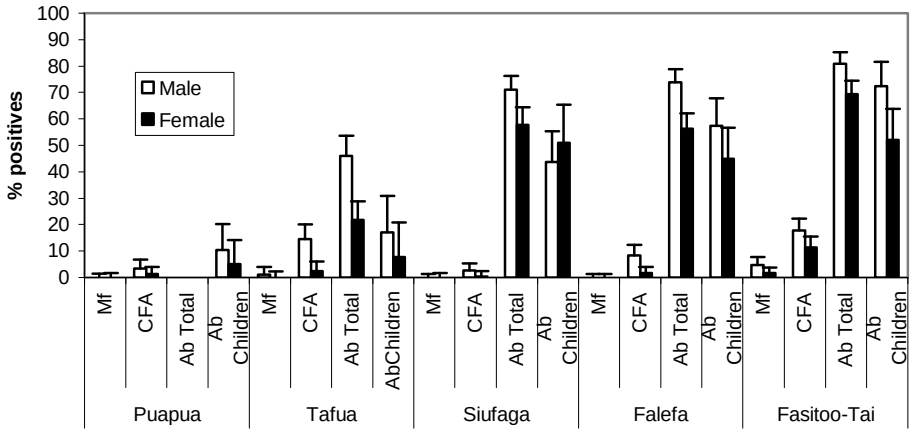
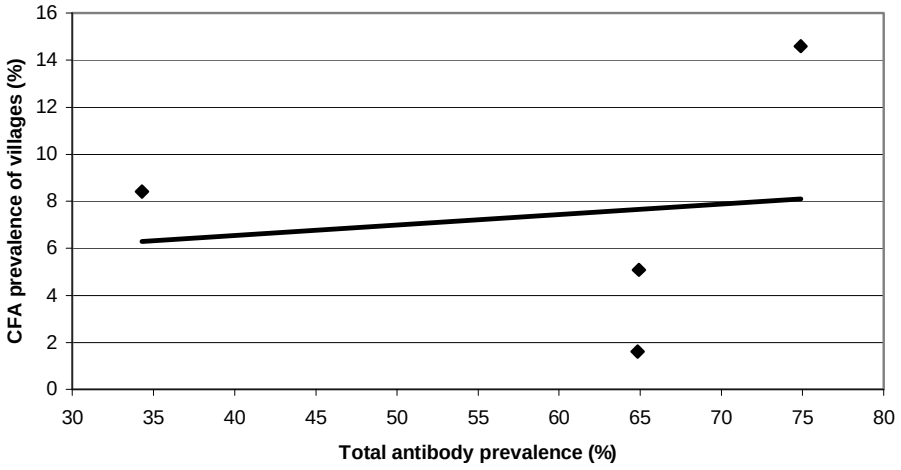


Figure 6.4: Gender-specific prevalence of Mf, CFA, and antibodies (Ab) for the total population and children. For each parameter for every village the prevalence was greater in males. In Puapua, antibodies were only measured in children.

(a)



(b)

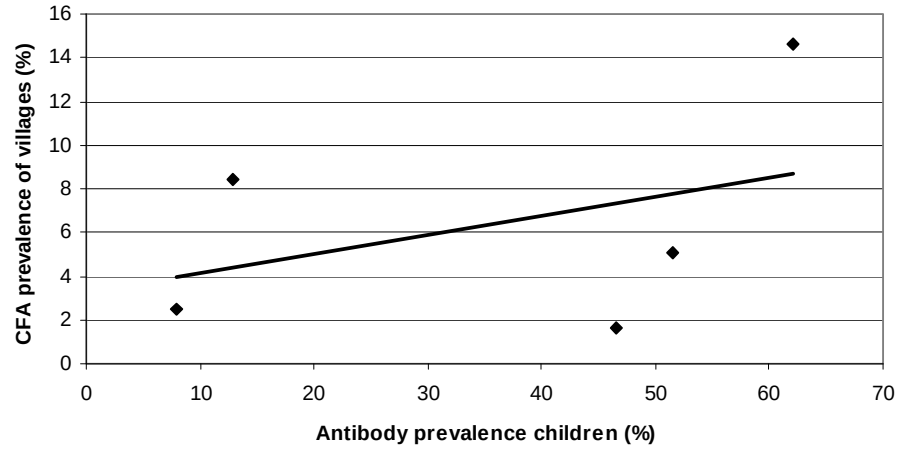


Figure 6.5: Correlation between CFA prevalence and (a) total antibody prevalence, and (b) antibody prevalence in children. It was observed that as the levels of CFA prevalence increased, so did the levels of antibody positivity for both the total population and children. However, this correlation did not reach statistical significance (For both: $r_s = 0.5$; $P = 0.391$).

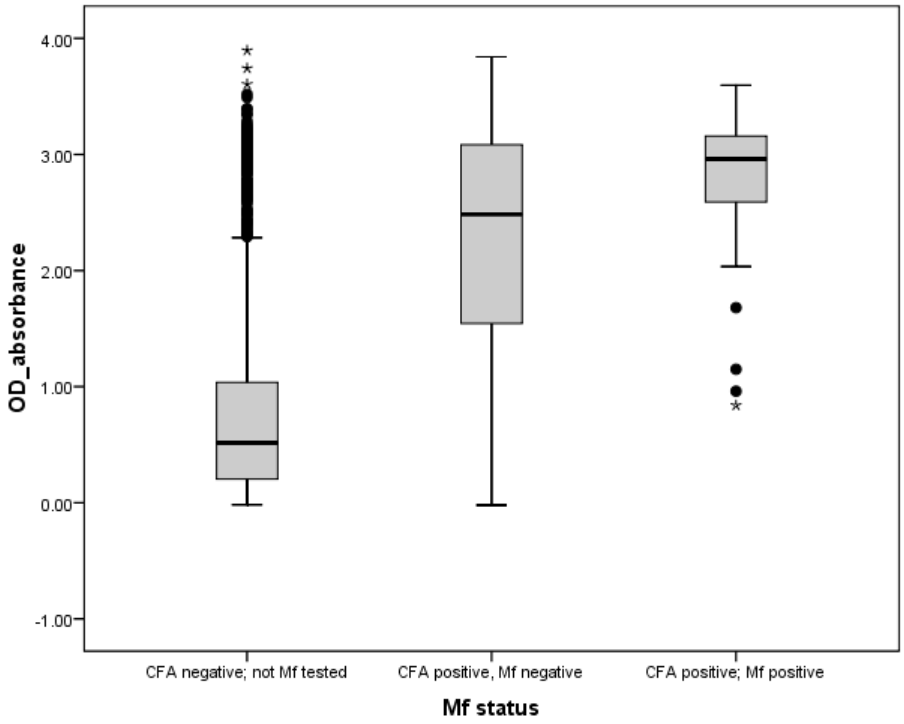


Figure 6.6: Mean OD absorbance values obtained from the Filariasis CELISA for the three groups of individuals: CFA negative, CFA positive and amicrofilaraemic, and, lastly, both CFA and Mf positive. The Mf positive individuals had a significantly higher OD absorbance value than the other two groups ($Z = 2.031$; $P = 0.038$). The CFA negative individuals had a significantly lower OD absorbance value ($Z = 13.71$; $P < 0.001$) than the other two groups.

6.5 DISCUSSION

Incorporation of antibody serology into the repertoire of LF diagnostic assays has been suggested as an earlier indication of resurgence and a sensitive method for identifying residual endemnicity (Ramzy, 2002; Durrheim ***et al.***, 2003; Rawlins ***et al.***, 2004; Grady ***et al.***, 2007; Weil and Ramzy, 2007). This suggestion was based on previous studies using the research-based Bm14 assay prototype and, in order for antibody serology to be included, the assay

must be standardised (Weil ***et al.***, 2010). The data described in the current research chapter clearly demonstrates the comparability of the Filariasis CELISA with these previous studies and the potential for antibody serology to identify areas of residual endemnicity.

The data indicated that ongoing transmission was occurring in all five villages as total CFA prevalence was greater than the defined threshold of 1% (PacELF, 2006). This was of a concern since Siufaga was chosen to represent a village where transmission was believed to be interrupted. The five villages chosen for this research differed in their infection prevalence levels, but overall antibody rates were higher than CFA rates, which in turn were higher than Mf rates. Thus, antibody results were comparable to previous studies using the research-based Bm14 assay prototype (Weil ***et al.***, 1999; Njenga ***et al.***, 2007a; Tisch ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009).

All 22 Mf positive individuals tested antibody positive, correlating with previous studies using the research-based Bm14 assay prototype (Ramzy ***et al.***, 1995; Weil ***et al.***, 1999). In addition, a significantly higher proportion of CFA positive individuals tested antibody positive (93.1%) than CFA negative individuals (57.1%). This agreed with results from the research-based Bm14 assay prototype in other epidemiological settings since the IgG_4 isotype is usually expressed during late exposure or pre-patent infection (Ottesen ***et al.***, 1985; Kurniawan ***et al.***, 1993; Haarbrink ***et al.***, 1995; Ramzy ***et al.***, 1995; Weil ***et al.***, 1999). Antibody titres, as measured by OD absorbance values

(Dylewski ***et al.***, 1984), were significantly higher in microfilaraemic individuals. Antibody titres were significantly low in CFA negative individuals. This concurred with previous studies by Njenga ***et al***. (2007), who demonstrated that the intensity of the anti-filarial IgG_4 response, using the research-based Bm14 assay prototype, correlated with antigenaemia. Overall, the performance of the Filariasis CELISA was favourable in comparison to the research-based Bm14 assay prototype.

It was hypothesised that ongoing transmission would correlate with exposure (antibody positivity) in children. The highest transmission was observed for Fasitoo-Tai, whereby significantly higher prevalence was observed for all three parameters measured, including CFA and antibody positivity in children. The villages with the lowest levels of transmission, Puapua and Siufaga, recorded a significantly lower CFA prevalence and, for Puapua, antibody prevalence in children. This data suggested an existence of a linear relationship between infection (defined by CFA positivity) and exposure (antibody positivity in children), but this did not reach statistical significance. Previous studies have noted parallel falls in antibody and CFA prevalence following MDA (Ramzy ***et al.***, 2006; Tisch ***et al.***, 2008; Weil ***et al.***, 2008), although levels of antibody can be highly variable and persist for years (Helmy ***et al.***, 2006). This reiterates the need to sample children as an index of ongoing transmission.

There was no correlation between CFA positivity and exposure in either Siufaga or Tafua. This may be because microfilaraemia in the population is

dissociated from antibody positive (exposed) children since the predominant vector in Samoa is the day-biting ***Ae. polynesiensis*** (Ramalingam and Belkin, 1964). Consequently, with the movement of people during the daytime, there is the potential for residents from other villages to be contributing to the ongoing transmission. Additionally, the inclusion of 10 year olds in the survey may have affected the data analysis since these individuals may have been born prior to the 1999 MDA. However, this was not the case (Table 6.3). Therefore, the correlation between CFA positivity and exposure in children requires further investigation using spatial statistic techniques (Chapter 7) and/or further epidemiological studies in other settings.

As mentioned in previous chapters, a surveillance strategy tailored to the Pacific has been proposed; "LF surveillance strategy for the PICT" (Huppatz, 2008). The strategy entails testing children aged between 5 and 6 years (first level school entrants) for CFA using the ICT, since CFA positive children are indicative of ongoing transmission (Rajasekariah ***et al.***, 1991; Weil ***et al.***, 1997; Sunish ***et al.***, 2002; Rawlins ***et al.***, 2004; PacELF, 2006; WHO, 2007a; Huppatz ***et al.***, 2008; Bal ***et al.***, 2009; Huppatz, 2008). It was also designed to identify areas of residual endemnicity, referred to as "hot spot surveys" (Huppatz, 2008). However, ICT testing in low prevalence settings has inherent problems. The sensitivity of the ICT when prevalence is low is unknown and, coupled with a lag phase of up to 12 months before an infected person tests positive, could be unsuitable for surveillance (Grady ***et al.***, 2007; Weil and Ramzy, 2007; Molyneux, 2009). In addition, it can take years for levels of CFA to rise to detectable levels (Lammie ***et al.***, 1998). In

high prevalence settings, children as young as 2 years have tested as CFA positive (Lammie ***et al.***, 1998). However, it is unknown how long it would take in a low prevalence setting (Lammie ***et al.***, 1998). This is because the host needs to be bitten thousands of times by infective mosquitoes to establish infection and, when prevalence declines, infective mosquito numbers decline (Hairston and de Meillon, 1968; Southgate, 1984; Ichimori and Crump, 2005).

If the above LF surveillance strategy for the PICT was implemented in the current research, four of the villages would have been detected as residual areas of endemnicity since > 0.1% of children were CFA positive. However, by the same definition, Siufaga would have been declared LF-free. This was not the case since the overall CFA prevalence exceeded 1%, which is defined as ongoing transmission (PacELF, 2006). This was reflected by high antibody prevalence in children. Thus, assaying CFA alone in children would not be adequate in this particular instance, highlighting the possibility for complementing the current strategy with antibody testing now that a standardised assay is available. The current strategy stipulated the need to be updated as new research came to light (Huppatz, 2008). Previous serological studies in higher prevalence settings have also suggested serology as an earlier indicator (Gao ***et al.***, 1994; Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Weil ***et al.***, 2000; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Tisch ***et al.***, 2008; Mladonicky ***et al.***, 2009).

The potential reservoir of ongoing transmission could be the older age group, in particular males, since CFA prevalence and antibody prevalence significantly increased with age and there was a higher measure of infection in males in all parameters. Increasing CFA and/or antibody prevalence with age has been noted previously, both in high prevalence and low prevalence settings (Mahoney and Kessel, 1971; Tisch ***et al.***, 2001; Terhell ***et al.***, 2002; Beuria ***et al.***, 2003; Njenga ***et al.***, 2007a; Mladonicky ***et al.***, 2009). This observation reiterates the importance of monitoring the incidence of new cases, possibly in young children, than prevalence overall.

Male predisposition to infection has been previously documented in Samoa, whereby males had a three to five-fold higher prevalence of Mf than females (Mahoney and Kessel, 1971; Ichimori ***et al.***, 2007b). These findings were similar to those observed in Kenya (Njenga ***et al.***, 2007a) and India (Ramaiah ***et al.***, 2007). Whether this is due to non-compliance, differential exposure, or the inability to mount a successful humoral immune response is unknown and warrants further investigation (Terhell ***et al.***, 2000a; Sahu ***et al.***, 2008). Previous studies have speculated that the likelihood of older men working on plantations may increase their chances of exposure and, thus, infection (Mahoney and Kessel, 1971). Also of concern was the significantly higher antibody prevalence in male children. Why male children are at a higher risk of exposure is unknown. Future MDA campaigns should target the male population as a potential reservoir of infection.

In Samoa, future studies are needed to ascertain if antibody exposure is dissociated from Mf index cases, using spatial studies, as well as identifying potential reasons for infection in this demographic group (Chapters 7 and 8). In other epidemiological settings, use of the antibody assay requires validation and mathematical modelling to determine the best sampling strategy if it were incorporated into the LF programme (Lammie ***et al.***, 2004; Michael ***et al.***, 2006b; Tisch ***et al.***, 2008). Thresholds and mathematical models could differ between countries, based on previous history of compliance, efficiency of vectors, history of movement of people, heterogeneic nature of LF and current endemnicity (Sunish ***et al.***, 2003; Talbot ***et al.***, 2008; Gambhir ***et al.***, 2010). The detection of ongoing transmission in Siufaga, originally thought to be LF-free, indicates the need for more stringent thresholds in Samoa where the efficient ***Ae. polynesiensis*** is endemic (Samarawickrema ***et al.***, 1985b; Burkot and Ichimori, 2002; Burkot ***et al.***, 2002; Burkot ***et al.***, 2006).

In summary, the key findings from the research described in this chapter were:-

1) Ongoing transmission of LF was observed for all villages, including Siufaga which was originally thought to be LF-free;
2) In three Samoan villages there was a correlation between transmission (Mf/CFA positivity) and antibody exposure in children;
3) Reservoir of infection appeared to be in the older age groups, particularly males;

4) Individuals Mf/CFA positive were likely to be antibody positive, similar to previous results with the research-based Bm14 assay prototype;
5) Most importantly, testing for CFA positivity in children alone, as per the current surveillance strategy, would not have identified Siufaga as an area of residual foci; and,
6) Complementing CFA positivity with antibody serology would solve this problem.

Thus it can be concluded that:-

1) Males need to be especially targeted for treatment to aid successful LF elimination;
2) The commercial Filariasis CELISA performed comparatively well to the research-based Bm14 assay prototype in the field setting using filter paper samples;
3) More stringent thresholds for defining a cessation of transmission is required in Samoa;
4) It is a necessity to include antibody serology, as measured with the Filariasis CELISA, in future surveys in Samoa since CFA positivity in children alone did not identify all residual areas of foci;
5) Again, inclusion of the Filariasis CELISA would be beneficial for the LF programme; and,

6) It is imperative to reassess the current surveillance strategy for the PICT and validate these findings in other epidemiological settings.

CHAPTER 7

EVIDENCE OF SPATIAL CLUSTERING OF RESIDUAL INFECTION OF LYMPHATIC FILARIASIS IN SAMOA

7.1 INTRODUCTION

During the initial stages of programmatic planning, spatial mapping was used on a large scale to predict areas of endemnicity in order for programme managers to target MDAs effectively and to plan elimination strategies (WHO, 1998a; Gyapong ***et al.***, 2002). The large-scale spatial mapping was based on either 50 km x 50 km or 25 km x 25 km grids, depending on the geographical area studied, since assessment of every community by means of filariasis surveys would be cumbersome and expensive (WHO, 1998a; WHO, 1998b). Therefore, by using these methods, an estimation of the distribution of filariasis could be ascertained, similar to that achieved in Africa with the OCP (Ngoumou ***et al.***, 1994; WHO, 1998b) and other NTDs (Brooker ***et al.***, 2009). Now that the prevalence of LF is declining, it is necessary to revisit spatial mapping in order to gain information regarding transmission patterns at finer scales since, as a mosquito-borne disease, LF is expected to show a high degree of heterogeneity over very small areas because of differences in vector distribution, flight ranges, population movement, and breeding habitats among other factors (Shope, 1999; Bhumiratana ***et al.***, 2005; Bockarie ***et al.***, 2009a; Gambhir ***et al.***, 2010). Spatial mapping not only allows quick identification of problem areas, as was the case with

seroprevalence mapping (Chapter 5), but also allows the use of spatial statistics to provide further insights into these patterns.

There is a difference between mapping the geographic distribution of LF (Gyapong ***et al.***, 2002; Melrose and Rahmah, 2006), and fine scale spatial mapping using spatial statistical software to understand transmission dynamics and identify "hotspot" clustering. Fine scale spatial mapping, also referred to as micro-spatial mapping, has been successfully implemented in a number of diseases to infer likelihood risks, risk factors, extent of the disease, vector control in vector-borne diseases, surveillance, and to gain information on targeting control efforts since fine scale spatial mapping is at a similar scale to that at which control measures are implemented (Brooker ***et al.***, 2006; Raso ***et al.***, 2006a; Raso ***et al.***, 2006b; Matthys ***et al.***, 2007; Raso ***et al.***, 2007; Pullan ***et al.***, 2008; Brooker and Clements, 2009; Clements ***et al.***, 2009; Eisen and Lozano-Fuentes, 2009). The understanding of transmission dynamics at the micro-spatial level would be extremely useful for effectively targeting residual LF endemic "hotspots" to understand the extent of their effect on the surrounding areas. It would also be useful in the future to delimit the areas around the zones of ongoing transmission that would require control efforts. This appears feasible in night-biting vector endemic areas since spatial clustering of LF has been known for a long time (Lammie ***et al.***, 1998; Weil ***et al.***, 1999; Terhell ***et al.***, 2000b; Alexander ***et al.***, 2003; Wahyuni ***et al.***, 2004; Washington ***et al.***, 2004).

To date, no spatial clustering of LF has been detected in areas where the day-biting ***Ae. polynesiensis*** vector is endemic (Cuenco ***et al.***, 2009; Mladonicky ***et al.***, 2009). ***Ae. polynesiensis***, a highly efficient vector when intensity of infection is low, is endemic in Samoa (Ramalingam and Belkin, 1964; Samarawickrema ***et al.***, 1985b; Snow ***et al.***, 2006). The persistent antigenaemia in Samoa drives the need to investigate these areas of residual foci to ascertain if infected individuals and/or exposed children are geographically related. This can be achieved with spatial statistical software such as SaTScan (Kulldorff ***et al.***, 2007).

SaTScan has been widely used in the epidemiological setting to study outbreaks (Sowmyanarayanan ***et al.***, 2008), viral diseases (Allepuz ***et al.***, 2008), other vector-borne diseases such as Rocky Mountain spotted fever (Adjemian ***et al.***, 2009), malaria (Brooker ***et al.***, 2004b; Zhang ***et al.***, 2008; Coleman ***et al.***, 2009), and even one study of LF (Washington ***et al.***, 2004). SaTScan has been favourably compared with other spatial methods, such as Bayesian Disease Mapping (Aamodt ***et al.***, 2006). The statistical computer programme functions to detect and evaluate clusters temporally, spatially, or in a space-time setting by scanning a window across space and/or time and noting the number of observed and expected cases or controls (Kulldorff, 2006). The window for spatial statistics, as used in this research, is circular or elliptical and multiple different window sizes are used. Using a spatial statistical theory, outlined in detail by Kulldorff (1997), a likelihood is calculated for each window and the most likely cluster correlates with the window with the maximum likelihood, which is assigned a P value based on

Monte Carlo hypothesis testing (Kulldorff, 2006). This powerful spatial statistical programme can test if a disease is randomly distributed or clustered over space while automatically adjusting for any underlying inhomogeneous spatial density or clustering due to chance (Kulldorff and Nagarwalla, 1995; Kulldorff, 2006).

As antibody production in response to LF exposure occurs during the first few years of life (Gao ***et al.***, 1994), children can serve as a sensitive indicator of LF transmission; because in the absence of transmission children should be antibody negative (Ramzy ***et al.***, 1995; Lammie ***et al.***, 1998; Lammie ***et al.***, 2004; Supali ***et al.***, 2004; Weil and Ramzy, 2007; Huppatz ***et al.***, 2008; Weil ***et al.***, 2008; Mladonicky ***et al.***, 2009). The persistent transmission in Samoa drives the need to investigate these areas of residual foci to define transmission patterns by ascertaining if infected individuals and/or exposed children are geographically clustered. Evidence of clustering would be useful for guiding surveillance efforts. Therefore, it was the aim of the research described in this chapter to establish if spatial clustering of LF existed in an ***Ae. polynesiensis*** endemic area and, if so, the relationship to exposed children. By doing so, the feasibility of using antibody serology as a means to complement future surveying strategies can be ascertained.

7.2 AIMS

The specific aims for the work described in this chapter are to:-

(1) Test for spatial clustering of residual infection;

(2) Test for spatial clustering of exposure in children ≤ 10 years; and,

(3) Investigate the relationship between infected individuals and exposed children and, by doing so, assess the feasibility of the CTS in Samoa.

7.3 MATERIALS AND METHODS

7.3.1 Study Area and Population

Same area and population as for Chapter 6 (Section 6.3.1 and 6.3.2).

7.3.2 Diagnostic Testing

Diagnostic testing is outlined in detail in Section 6.3.2. Test results from each participant (Section 6.4.1) were used in the spatial analyses.

7.3.3 Spatial Data Collection

Every household within the village was mapped using a GPS handheld device and assigned a unique identifier. The GPS used was a handheld eTrex Legend (Garmin International Ltd., USA). Although the unit

specifications for accuracy were < 15 m Root-Mean-Square (RMS) (95% of the readings within 15 m radius), it was found that variations in GPS readings could be obtained over 1-2 m. To obtain this accuracy GPS readings were measured when there was no cloud cover. The unit had a 12-channel all-in-view tracking and National Marine Electronics Association (NMEA) 0183 GPS protocol.

When a family had more than one living area on their land, a reading was taken from the centre of their property. If an individual had multiple residencies in different parts of the village, their place of residence was defined as the place where they slept. GPS measurements were converted into decimal degrees for statistical analysis using SaTScan Version 7.0 (Kulldorff ***et al.***, 2007). SaTScan can either be set for a Poisson or Bernoulli population. The Bernoulli model was used since there was a definitive number of participants in the study and it is more appropriate for case/control data (Brooker ***et al.***, 2004b). For accurate mapping, the decimal degrees were further converted into a projected datum (WGS84 Zone 2S) using the GIS package ArcGIS 9.3 (ESRI, 2008).

7.3.4 Statistical Analysis

Spatial clustering was assessed using SaTScan (Kulldorff, 1997). The unit of analysis was the individual since SaTScan can recognise each resident within a geographical location, such as a household, as a discrete entity. Therefore, instead of defining an entire household as a “case”, if an individual

residing there was infected, each household location stated the number of "controls" and "cases". Different weightings occur depending on the number of occupants and the ratio of controls to cases within that household. For this research a circular window was used for spanning the study site, as previously used in mosquito-borne spatial studies (Brooker ***et al.***, 2004b; Washington ***et al.***, 2004; Zhang ***et al.***, 2008; Coleman ***et al.***, 2009). The circular window centres on a number of possible grid locations within the study site, and the size of the window varies from a radius of 0 m to a pre-defined upper limit. The upper limit was set so as never to include more than 50% of the study population since a circle to include more than 50% of the individuals in the study site would be inappropriate for determining significance of relative risk (RR) of disease (Kulldorff and Nagarwalla, 1995). A window set at no more than 50% of the study population is also useful for detection of small and large clusters (Kulldorff and Nagarwalla, 1995). SaTScan also allows the user to set the upper limit based on geographical distance. This was deemed inappropriate for this particular study since in areas where intense transmission of LF could be occurring it would be expected that there would be greater than one index case. This widens the possible geographical area and thus I did not want to set biased upper limits for SaTScan searching. Secondly, without entomological data it was difficult to ascertain the geographical area that would be affected by a single mosquito.

Significant clusters were defined with a central point (with latitude and longitude readings) and a radius (m) of how far geographically the cluster

extends from this central reading. Any persons within this cluster area during peak mosquito biting times were at a statistically higher risk of becoming a case than if they were elsewhere. Clusters identified by the software that did not reach the $P < 0.05$ significance level were not included. If the analysis identified significant primary "most likely" clusters as well as over-lapping secondary clusters that were significant, only the "most likely" cluster was included in the results.

The two scenarios explored by virtual analysis were "community based", whereby exposure could be occurring around the household, or "school based" whereby a child is being exposed whilst at school.

The case definitions used for both scenarios were:

(1) To identify microfilariae clustering: microfilariaemics as the case, every other individual in the house defined as a control;

(2) To identify antigen clustering: CFA positives as the case, every other individual in the house defined as a control;

(3) To identify antibody clustering: antibody positive child aged 10 years and below defined as the case, every other individual in the house defined as a control;

(4) To identify clustering of antigen and/or antibody cases: the cases were both CFA positive individuals of any age and/or antibody positive child aged 10 years and below, every other individual in the house defined as a control; and,

(5) To identify clustering of Mf- and/or antibody positive cases: the cases were both microfilaraemic individuals and/or antibody positive children aged 10 years and below, every other individual in the house defined as a control.

Clusters identified for definitions 1-3 will be termed ‘cluster’. Clusters identified from definitions 4-5 will be termed ‘dual clusters’ and refer to a cluster of two properties complementing each other, rather than representing two clusters merged together.

All villages were mapped using ArcGIS 9.3 (ESRI, 2008). Using the radius and centroid outputs from the SaTScan analysis, clusters were included on the map, to scale, using the software extension XTools Pro V 4.1.

7.4 RESULTS

7.4.1 Spatial Clustering

The RR and size of each primary cluster is outlined in Table 7.1. The RR defined the likelihood of an individual within this area of being infected or exposed, depending on the analysis explored from the methods section.

Table 7.1: Summary spatial data of the five villages examined. Data presented here include whether a spatial cluster was observed (Yes (Y) or No (N)), the RR of individuals living within the cluster, and radius of cluster in metres (Rad).

Island	Village	Number of HH	"Community based"					"School based"		
			Mf	CFA	Ab	Mf and Ab	CFA and Ab	Ab	Mf and Ab	CFA and Ab
Upolu	Fasitoo-Tai A = 6.8 km^2	92	N	Y RR = 3.881 P = 0.001 Rad = 1160 A = 4.2 km^2	Y RR = 2.371 P = 0.004 Rad = 1160 A = 4.2 km^2	Y RR = 2.517 P = 0.001 Rad = 1340 A = 5.6 km^2	Y RR = 2.799 P = 0.001 Rad = 640 A = 1.3 km^2	Y RR = 7.292 P = 0.001 Rad = 80 A = 0.02 km^2	Y RR = 4.736 P = 0.001 Rad = 1380 A = 6 km^2	Y RR = 1.983 P = 0.001 Rad = 280 A = 0.2 km^2
	Falefa A = 6.4 km^2	70	N/A	N	N	N/A	N	Y RR = 6.038 P = 0.001 Rad = 220 A = 0.15 km^2	N/A	Y RR = 2.781 P = 0.001 Rad = 1150 A = 4.1 km^2
	Siufaga A = 4 km^2	75	N/A	Y RR = 82.167 P = 0.006 Rad = 0 A = 0	N	N/A	N	Y RR = 11.290 P = 0.001 Rad = 0 A = 0	N/A	Y RR = 7.988 P = 0.001 Rad = 60 A = 0.01 km^2
Savai'i	Puapua A = 6.2 km^2	88	N/A	Y RR = 14.167 P = 0.009 Rad = 470 A = 0.7 km^2	N	N/A	Y RR = 6.833 P = 0.003 Rad = 700 A = 1.5 km^2	Y RR = 12.715 P = 0.027 Rad = 0 A = 0	N/A	Y RR = 24.556 P = 0.007 Rad = 0 A = 0
	Tafua A = 19.5 km^2	62	N	N	N	N	N	N	N	N

A = area in km^2, estimated by ArcGIS software V 9.3.
N/A = not applicable
HH = household

7.4.1.1 "Community based" scenario

Clustering of cases is summarised in Table 7.1. Households containing infected (CFA positive) individuals showed spatial clustering in the villages of Fasitoo-Tai, Siufaga and Puapua (Figure 7.1a, 7.1c, and 7.1d). LF exposure, defined by antibody positive children, was only evident in Fasitoo-Tai (Figure 7.1a and 7.1b).

Dual clustering, including households with infected individuals and antibody positive children, was observed in Fasitoo-Tai and Puapua (Figure 7.1a and 7.1d). Mf positives were detected in Tafua and Fasitoo-Tai. Only in Fasitoo-Tai was there a dual cluster between households with Mf positive individuals and antibody positive children.

No significant spatial patterns were identified for either Falefa or Tafua, each with a CFA prevalence of 5.1% and 8.4% respectively (Table 6.3), although it was observed that a higher number of individuals with CFA lived within approximately 400 m of the coast for both villages (Figures 7.1e and 7.1f).

7.4.1.2 "School based" scenario

Different spatial patterns from the "community based" scenario were observed for the "school based" scenario. For four villages, excluding Tafua, a spatial cluster of antibody positive children was observed incorporating either one or two primary schools (Table 7.1 and Figure 7.2). In Fasitoo-Tai,

Siufaga and Falefa this spatial cluster extended to a dual cluster when infected individuals were included in the analysis (Figure 7.2a, 7.2c and 7.2e), thus widening the geographical limits of the hypothetical area of transmission. In Puapua, this dual cluster existed separate to the cluster of antibody positive children (Figure 7.2d). Lastly, again, no spatial clustering was observed for Tafua (Table 7.1 and Figure 7.2f).

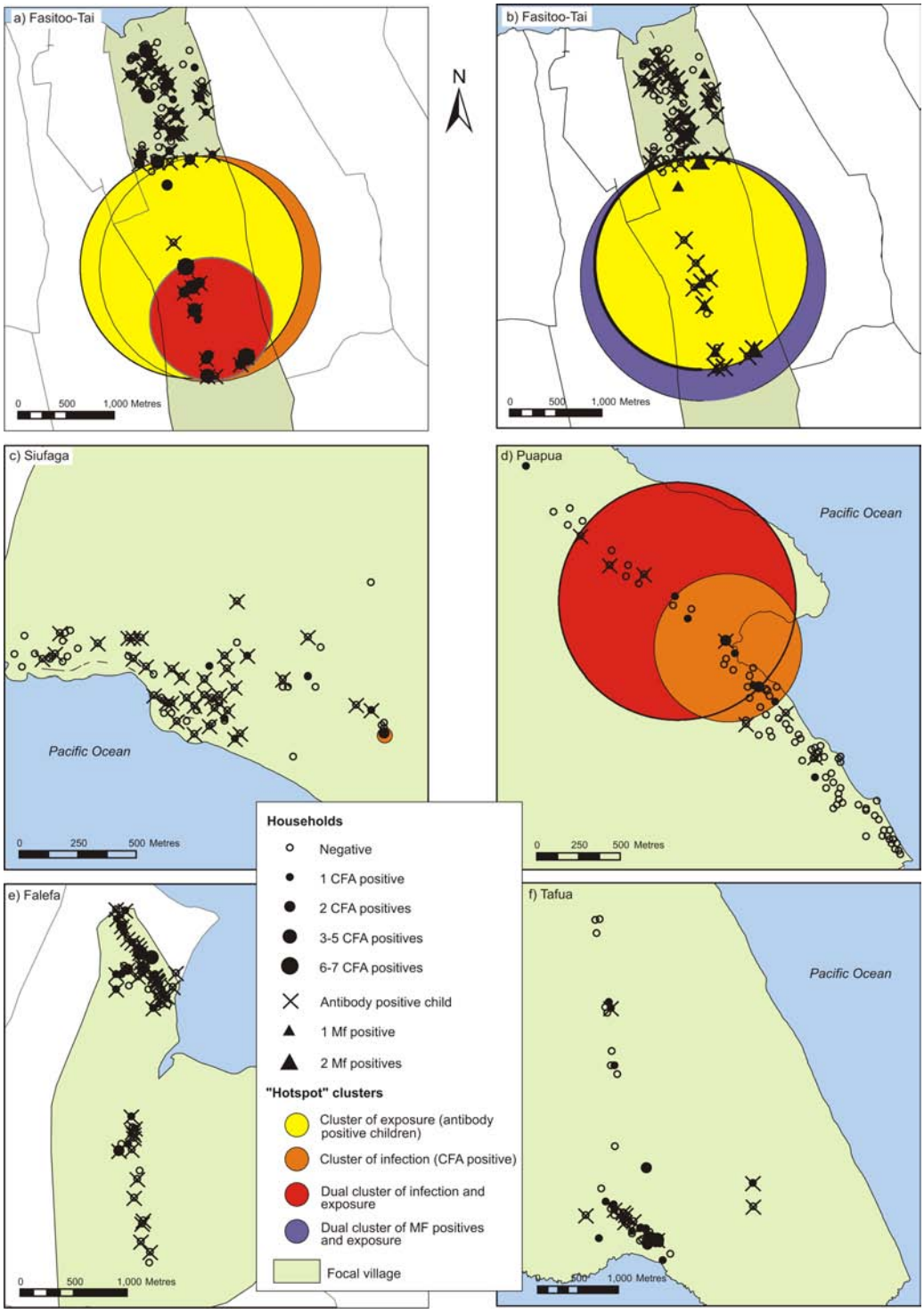


Figure 7.1: The "community based" scenario highlighting the spatial clusters of LF exposure and/or infection in each of the five villages: (a) Fasitoo-Tai, (b) Fasitoo-Tai, (c) Siufaga, (d) Puapua, (e) Falefa, and (f) Tafua. There were four types of clusters identified including households with CFA positive individuals, with children exposed to LF, a dual cluster of CFA positive individuals and exposed children, and a dual cluster of Mf/CFA positive individuals and exposed children.

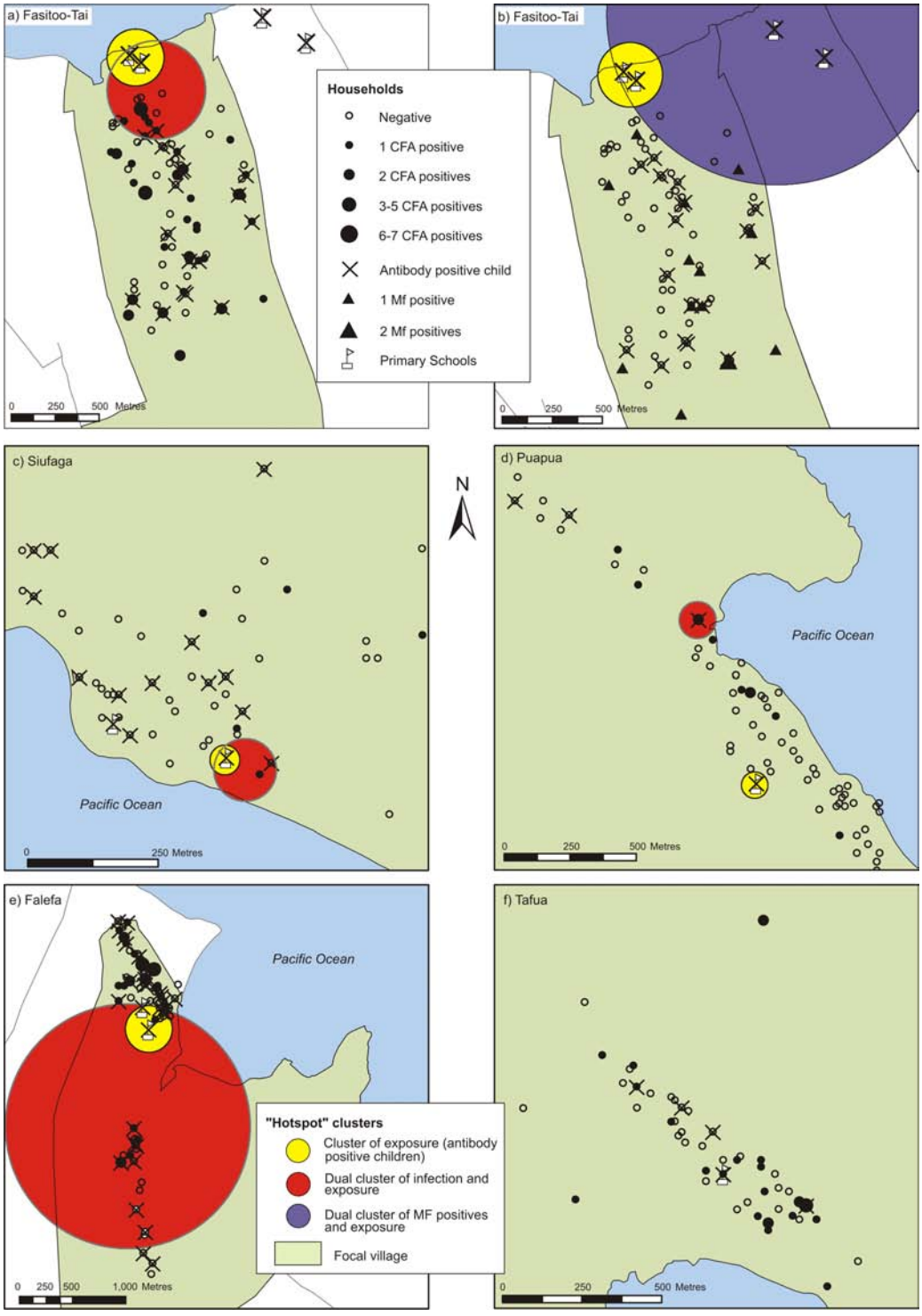


Figure 7.2: The "school based" scenario highlighting the spatial clusters of LF exposure and/or infection in each of the five villages: (a) Fasitoo-Tai, (b) Fasitoo-Tai, (c) Siufaga, (d) Puapua, (e) Falefa, and (f) Tafua. There were four types of clusters identified including households with CFA positive individuals, with children exposed to LF, a dual cluster of CFA positive individuals and exposed children, and a dual cluster of Mf/CFA positive individuals and exposed children.

7.5 DISCUSSION

This research is the first evidence of spatial clustering of LF cases in an ***Ae. polynesiensis*** vector endemic area and holds profound implications for the future of LF control and surveillance strategies in Samoa and, indeed, other countries where this highly efficient vector is endemic.

Previously it was believed that, because the vector is predominantly a day-biter, the usefulness of vector control was limited and it would require countrywide MDAs to control persistent residual foci of LF (Burkot ***et al.***, 2006; Bockarie ***et al.***, 2009a). This is because spatial clustering of LF in these areas had not been identified and the lack of clustering was concluded to be due to the mobility of infected individuals during the day (Mladonicky ***et al.***, 2009; Stoddard ***et al.***, 2009). This would make control difficult. On the contrary, the current study has demonstrated that the household and/or primary school of attendance could still serve as a major site of LF exposure. This is because significant clustering was observed for both the "community based" analysis and the "school based" analysis.

The potential for both "community based" and "school based" clustering to be observed could relate to the vectors in Samoa; ***Ae. polynesiensis*** (day-biter) and ***Aedes samoanus*** (night-biter) (Samarawickrema ***et al.***, 1985b). Hypothetically, the presence of spatial clustering around schools, in the "school based" analysis, could be due to the day-biting tendencies of ***Ae. polynesiensis*** (Jachowski, 1954; Ramalingam, 1968; Rakai ***et al.***, 1974;

Bockarie ***et al.***, 2009a). The fact that clustering around households in the "community based" analysis was also observed is noteworthy.

Ae. polynesiensis could potentially contribute to biting around the household in the "community based" scenario too since biting peaks just after sunrise until approximately 0800 hours and again before sunset when individuals are likely to be at home (Jachowski, 1954; Ramalingam, 1968; Rakai ***et al.***, 1974; Bockarie ***et al.***, 2009a). This would explain the presence of two discrete clusters observed for the "school based" scenario in Puapua. Secondly, ***Ae. samoanus***, although a minor vector and relatively inefficient in transmission, could also contribute to LF exposure at night-time, whilst an individual sleeps at home (Ramalingam, 1968; Samarawickrema ***et al.***, 1985b). However, without an in-depth entomological study within these communities, conclusions as to specific vector contributions to the transmission dynamics cannot be ascertained. Conclusions can only be drawn for the presence of spatial clustering.

Evidence of household clustering of LF in a night-biting vector endemic area was reported in 1974 (Walter, 1974). Following this research, links to familial and genetic predisposition have been explored, with evidence that certain genes may be the cause of increased susceptibility to LF (Ottesen ***et al.***, 1981b; Lammie ***et al.***, 1991; Terhell ***et al.***, 2000b; Wahyuni ***et al.***, 2004). Other studies have found that infection with either parent increases the risk of infection for children (Das ***et al.***, 1997; Alexander ***et al.***, 1998) due to shared environmental factors (Weil ***et al.***, 1999) since filariasis could be transmitted

in and around houses depending on the vector present (Gad ***et al.***, 1994). These studies highlighted the need to treat other members of the household if one member was infected. Subsequent studies recognised an increased risk of exposure to surrounding households, by measuring anti-filarial IgG_1, and concluded that the nearest neighbours should also be treated (Washington ***et al.***, 2004). The current data agrees with this to some extent, but the spatial clusters observed extend past the nearest neighbour suggesting a larger geographical area of potential exposure.

The extent of the affected area emphasises the need to target treatment in Samoa effectively to eliminate these residual foci. The total area required to target treatment differed in each village, possibly due to the intensity of transmission, since it was clear that the village with the highest prevalence (Fasitoo-Tai) had a larger radial risk of CFA positive individuals and antibody positive children. When the analysis included both CFA positive individuals and antibody positive children a dual cluster was observed with a smaller geographical area. This could potentially represent an area of intense transmission. Rising IgG_4 levels are associated with late exposure or early pre-patent infection (Ottesen ***et al.***, 1985; Hussain ***et al.***, 1987; Kwan-Lim ***et al.***, 1990; Hitch ***et al.***, 1991; Estambale ***et al.***, 1994b; Mohanty ***et al.***, 2007), further evidence that effective targeted treatment is needed in these areas.

The data presented here also highlights the need to move away from the original spatial mapping (50 km x 50 km and 25 km x 25 km grids) proposed by the WHO during the beginning of programme mapping, to much smaller

areas spanning 1 km^2. This concurs with previous research in PNG which demonstrated that the spatial correlation of ***W. bancrofti*** Mf density reduced by half over 1.7 km (Alexander ***et al.***, 2003). Furthermore, in India, it was recommended to reduce the 25 km x 25 km WHO grids to < 10 kms^2 (Srividya ***et al.***, 2002). These studies correlate with new findings highlighting the heterogeneity of LF transmission over small geographical areas (Gambhir ***et al.***, 2010). By reducing the area analysed for spatial mapping, spatial patterns around households can be ascertained, such as the case with Samoa. This allowed detection of ongoing LF transmission in the chosen study areas.

Surveillance strategies rely on the accurate and sensitive detection of LF transmission. The current study suggests the use of antibody serology in children since a significant relationship was observed between this parameter and Mf/CFA positive individuals within the community. Close contact testing, as per the LF surveillance strategy for the PICT, involves tracing the potential source of infection from the child's home by testing surrounding households (24 houses or a radius of 200 m) (WHO, 2007a; Huppatz ***et al.***, 2009). The results from the current research suggest the potential for children to be exposed to LF either during the day, whilst at school, or when at home. Therefore, in Samoa, tracing the potential source of infection should occur both from the child's home and the primary school of attendance, which will affect the current surveillance strategy.

Currently, close contact testing, as per the LF surveillance strategy for the PICT, has been advised for up to 200 m from the infected child's home to find and treat the Mf positive individual (Huppatz ***et al.***, 2009). The results from the current study extend past this arbitrary figure and also suggest that additional close contact testing may be required radiating out from the household of the Mf positive individual once identified, since dual clusters were observed between Mf positive individuals and exposed (antibody positive) children. For Samoan villages in the current study, a much wider geographical limit of clustering was recognised, extending to over 1 km in Fasitoo-Tai. It could be speculated that the wider radii obtained in the current study may be due to the presence of > 1 Mf positive individual contributing to the ongoing transmission since CFA prevalence exceeded 1% in all villages studied. This would widen the limits of the geographical area where residents are potentially exposed. Consequently, it could be recommended that when CFA prevalence exceeds 1% to extend the suggested radius of 200 m if following the current LF surveillance strategy for the PICT (Huppatz, 2008). Especially since in the two villages with the lowest CFA prevalence the significant spatial clusters recorded a radius of up to 470 m. The statistically significant smaller cluster of CFA positive individuals in Siufaga (0 m), was comparable to spatial analysis research in other mosquito-borne diseases such as dengue (Morrison ***et al.***, 1998), where clustering was concluded to be due to multiple feeding behaviours of mosquitoes around the homes.

Another crucial finding from the current study was the dual clustering of infected (CFA positive) and exposed (antibody positive) as well as Mf positive and exposed (antibody positive) children. This favours the potential for antibody serology to be incorporated into the surveillance strategy since CFA testing alone may not be adequate (Chapters 5 and 6). Consequently, antibody serology may be the better diagnostic assay choice, providing an earlier warning of potential resurgence. This requires further study in other epidemiological settings both in the Pacific and other LF endemic regions of the world. Children would represent the prime cohort as a measure of incidence, which has proven useful in other parasitic diseases, including the OCP (Massara ***et al.***, 2006; Lindblade ***et al.***, 2007; Enk ***et al.***, 2008).

In the current research the search window to identify spatial clustering was circular, which has been successfully used in a number of infectious diseases (Cousens ***et al.***, 2001; Fevre ***et al.***, 2001; Ghebreyesus ***et al.***, 2003) and mosquito-borne diseases (Brooker ***et al.***, 2004b; Washington ***et al.***, 2004; Zhang ***et al.***, 2008; Coleman ***et al.***, 2009). Obviously a true cluster could be any shape, including rectangular or elliptical. However, by pre-selecting a different shape, other than circular, SaTScan has a reduced ability to detect other shaped clusters (Brooker ***et al.***, 2004b). Another advantage for using the circular scan statistic is that it is isotropic with respect to a rotation of the map (Brooker ***et al.***, 2004b; Coleman ***et al.***, 2009). Unfortunately, this is not the case with the elliptical scan statistic, unless all angles are considered, which is difficult for computational reasons (Brooker ***et al.***, 2004b; Kulldorff ***et al.***, 2006). Also, the elliptical search window cannot be used for latitude and

longitude coordinates, instead the coordinates must be first projected onto a planar map (Kulldorff, 2006). A comparative study, employing both a circular shape and ellipse, concluded that the exact choice of shape was not of critical importance for identifying clustering, but rather to allow the scanning window to cross a number of centre points and sizes (Kulldorff ***et al.***, 2006). Based on these conclusions, it was deemed appropriate for the current study to use a circular process for the identification of clustering.

The differences in spatial cluster radii between the five villages could be due to vector distribution (Samarawickrema ***et al.***, 1987a), the geographical layout of a village (coastal vs. inland) (Rakai ***et al.***, 1974), differing environmental circumstances (Mahoney and Kessel, 1971), differences in socioenvironmental composite risk indicators (Bonfim ***et al.***, 2009), or the intra-community variations in transmission observed over small distances such as a few households (Ramzy ***et al.***, 1994; Tisch ***et al.***, 2001; Washington ***et al.***, 2004; Gambhir ***et al.***, 2010). This may relate to the flight dynamics of the vector, which have been shown to range from a weak flier, only being able to fly a few hundred metres (Reiter ***et al.***, 1995), to up to distances of 800 m in the extreme (Honorio ***et al.***, 2003).

7.5.1 Future Directions and Conclusions

Environmental factors, such as wind patterns, could potentially affect the size and geographical position of the cluster. Using spatial analysis software, maps of geographical features of interest can be overlain onto already

existing GPS mapping of households, to identify any common patterns such as elevation and rainfall. Elevation has been identified as a limiting factor for LF transmission since there is an inverse relationship between the infectivity of a mosquito and increasing altitude (Attenborough ***et al.***, 1997). This is related to the cooler temperatures at higher altitudes. The maximum elevation cut-off for ***W. bancrofti*** transmission was found to be 1000 m, whereby most LF cases are confined to lowland and coastal areas (Hawking and Denham, 1976; Chanteau and Roux, 2008). Therefore, integration of spatial mapping with climate-based risk models could be beneficial for Samoa, as has been shown in other disease models (Brooker, 2007; Simoonga ***et al.***, 2009).

Identification of spatial clustering in Samoa is not only promising in terms of future surveillance and targeted treatment, but also for the potential for incorporation of vector control strategies. Vector control in the past was thought infeasible because of the day-biting tendencies of the vector, lack of spatial clustering of cases, and location of breeding habitats which are preferentially outdoors (Ramalingam, 1968; Samarawickrema ***et al.***, 1987a; Burkot ***et al.***, 2002; Bockarie ***et al.***, 2009a; Mladonicky ***et al.***, 2009). However, recent studies have demonstrated the potential for source reduction campaigns for both wet and dry seasons in American Samoa, where ***Ae. polynesiensis*** is also endemic (Burkot ***et al.***, 2007; Lambdin ***et al.***, 2009). Despite this, conclusions from this study were that without evidence of spatial clustering of breeding sites or LF cases, source reduction campaigns need to target the entire village (Lambdin ***et al.***, 2009; Mladonicky

et al., 2009). On the contrary, the current research suggests that source reduction could begin around households that are part of the identified cluster, highlighting the potential for incorporation of source reduction vector campaigns in Samoa. However, further temporal and entomological studies in Samoa would be required.

It was a concern that the "school based" scenario may bias the data since placing the children in their respective schools as their "household" for data analysis could result in a cluster of exposure by default. However, excluding Tafua, there were ≥ four primary or pre-schools recorded for each village, resulting in a broad geographic distribution of cases and controls. There was only one primary school attended in Tafua and no spatial clusters were identified here, possibly because Tafua was the village encompassing the largest geographical area (19.5 km^2). Higher antibody prevalence in children did not appear to influence the detection of clusters since Puapua had a lower antibody prevalence than the four other villages and clusters were still detectable. The lack of clustering observed for Falefa in the "community based" scenario may be due to the relative contributions of the vectors to transmission since clustering was observed for the "school based" scenario. Without entomological studies this cannot be ascertained.

Collectively, these promising findings are the first evidence of spatial clustering of LF in a day-biting ***Ae. polynesiensis*** vector endemic area. This research provides important information to give health personnel a starting point for finding Mf positive cases as the root of the residual endemnicity or

during surveillance, allow for targeted treatment efforts, and potentially incorporate vector control campaigns. This would help staff revise current policies to include: 1) treating households within a certain radius from the index case, and, 2) possible introduction of vector control, which has been shown to a) potentially reduce the number of years of MDA required to eliminate transmission, b) be necessary in those areas where ***Ae. polynesiensis*** is endemic, c) be necessary in areas with high vector density, and, d) lessen the likelihood of acquiring drug resistance (Molyneux ***et al.***, 1999; Reuben ***et al.***, 2001; Burkot ***et al.***, 2002; Das and Subramanian, 2002; Michael ***et al.***, 2004; Burkot ***et al.***, 2006; Das and Vanamail, 2008; Kyelem ***et al.***, 2008; Lambdin ***et al.***, 2009). Lastly, the spatial relationships observed between antibody positive children and Mf positive or CFA positive individuals enable the opportunity to further explore the use of antibody serology in surveillance strategies. Future research could also be conducted in a different ***Ae. polynesiensis*** endemic country, such as French Polynesia, to validate these results. Further research is also important to ascertain if infected individuals living within these spatial clusters are systematically non-compliant (Chapter 8).

In summary, the key findings from the research described in this chapter were:-

1) The first evidence of spatial clustering of LF cases in an ***Ae. polynesiensis*** vector endemic area;
2) Spatial clustering occurred for both the “community based” and “school based” scenarios;

3) The geographical extent of the cluster was related to the prevalence in each village;
4) Clustering was observed in the “LF-free” village of Siufaga; and,
5) Dual clusters were observed for Mf/CFA positive individuals with exposed children for both analyses.

Thus it can be concluded that:-

1) The extent of the affected area emphasises the need to target treatment in Samoa effectively to eliminate these residual foci;
2) The geographical size of the control area will differ depending on the prevalence. This is important information for the LF programme managers in Samoa;
3) Children are likely to be exposed both at home and at school during the day. Thus, current strategies should be revised to include close contact testing around a child’s school;
4) The geographical extent of close contact testing will depend on the LF prevalence in the community and should be revised accordingly;
5) Current policies should be revised to include vector control such as source reduction campaigns;
6) There is a spatial link between people infected and children exposed to infection. This has profound implications for future surveillance;
7) Clustering in Siufaga reiterates the importance of the inclusion of antibody serology into the LF programme; and,

8) The Filariasis CELISA has promising application as a diagnostic tool in the LF programme.

CHAPTER 8

EXPLORATORY STUDY INVESTIGATING FACTORS INFLUENCING COMPLIANCE WITH MASS DRUG ADMINISTRATION IN SAMOA

8.1 INTRODUCTION

In 1999, following the initiation of GPELF, 16 of the 22 countries in the PICT falling under the jurisdiction of PacELF, were classified as endemic for Bancroftian filariasis (WHO, 2007a). Five of these, including Samoa, have completed at least the obligatory five rounds of MDA and were recently assessed for their post-campaign prevalence (Huppatz ***et al.***, 2009). The investigation revealed a low level persistence of LF transmission in Samoa (Chapters 5, 6 and 7) (Huppatz ***et al.***, 2009), consistent with earlier reports of resurgence in Samoa (Ichimori, 2001), and thought to be the result of residual groups of non-compliant, infected individuals (Huppatz ***et al.***, 2009). This reported persistence of transmission in Samoa emphasises the need for community surveys to ascertain levels of MDA compliance and general awareness of LF.

Due to this need for further information and understanding about MDA compliance, the present chapter is an exploratory study which assesses the links between levels of knowledge and awareness of LF and MDA compliance in a group of infected individuals. Additionally, since ongoing educational campaigns may be required to ensure that the younger

population remains aware of the importance of MDAs and disease, a group of children born after the formation of PacELF (7 to 10 years), was also assessed for these characteristics.

8.2 AIMS

The specific aims for the work described in this chapter are to:-

(1) Investigate the knowledge base of individuals testing CFA positive (Chapter 6);

(2) Investigate the knowledge base of children aged 7 to 10 years; and,

(3) Investigate whether those systematically non-compliant infected individuals were more likely to reside within spatial clusters of infection identified in Chapter 7.

8.3 MATERIALS AND METHODS

8.3.1 Study Population

Data were available for five villages where epidemiological prevalence studies were conducted (Chapters 6 and 7). In these villages, television (TV) and radio are key media for the provision of information to the population generally, i.e., the study area is not isolated from information about LF and LF elimination programmes, as are more remote parts of the country.

Data were available for two groups in the population:

i) CFA positive group: Individuals who tested positive for CFA during the epidemiological study were asked to participate in a brief survey, regardless of their age (Table 8.1a); and,

ii) Children aged 7 to 10 years: All children aged between 7 and 10 years were also asked, through their parents and carers, to participate in a similar brief survey (Table 8.1b).

Ongoing transmission in the five study villages was defined in two ways:

1) Based on the detection of Mf positives (WHO, 2005); residents from villages with Mf positive people were classified as “high Mf prevalence” and those from areas where no Mf people were detected were classified as “low Mf prevalence”; and,

2) Based on the presence of CFA positive children (WHO, 2005); residents from villages where CFA prevalence in children exceeded 1% were classified as “high prevalence” and those where CFA prevalence was below 1% were “low prevalence”. The threshold set by the WHO for ongoing transmission is ≥ 0.1% of CFA positive children born after the initiation of effective MDAs (WHO, 2005). For this study a slightly higher threshold of ≥ 1% was set to be confident that transmission was occurring. The areas are referred to as “high CFA prevalence” or “low CFA prevalence”.

8.3.2 Defining MDA Compliance

Individuals who had participated in ≥ 1 MDA were considered compliant, regardless of whether they participated every year. Those who did not participate in any MDA were recorded as "systematically non-compliant". This definition was crucial since only those systematically non-compliant are deemed the true reservoirs of infection and contribute to persistent transmission (Boyd ***et al.***, 2010). This is because those who are sporadically non-compliant would have received treatment at some point in time (El-Setouhy ***et al.***, 2007).

Since retrospective studies have limitations concerning recall bias, in order to prompt a participant's memory (especially the children), participants were shown the tablets when asked if they participated in MDAs. Most importantly, participants were asked if they "swallowed" the tablets given to them since there have been differences noted between the distribution of tablets and their consumption (Gunawardena ***et al.***, 2007; Kumar ***et al.***, 2009).

8.3.3 Questionnaire

Prototype questionnaires underwent pilot testing prior to their application in the field. Personnel with a long-standing association with public health programmes edited the questionnaires based on their experiences and cultural understanding. Trial interviews were conducted with volunteers who

were either members of staff or younger members of their respective family. From this, minor changes were made and both questionnaires were translated into Samoan. Samoan staff members from the Ministry of Health generously volunteered to assist the researchers to conduct the interviews. Following the interview, Mrs Maiava (LF programme manager, WHO, Samoa) translated the questionnaire into English.

Questions asked of CFA positive individuals were designed to ascertain their level of knowledge of LF and to assess contributing factors for MDA non-compliance. The questions were:

1) Do you swallow the tablets from Mass Drug Administrations?
2) Have you heard of lymphatic filariasis?
3) Do you know how lymphatic filariasis is transmitted?

   If 'yes', how?
4) Do you use protection against mosquitoes?

   If 'yes', what type?
5) Have you tested positive to infection previously?

   If 'yes', when (which year/s)?

The questionnaire designed for the children aged 7 to 10 years was a truncated version of the questionnaire for CFA positive adults and older children to predominantly ascertain the level of knowledge and MDA participation. The questionnaire was administered by Samoan staff members of the Ministry of Health. Every child residing in the village was asked to participate. Children were either interviewed in their place of residence or at

their respective primary school if they attended. Prior to the interview process verbal consent was obtained from a parent or guardian. In addition, for those children attending school, permission was also sought from the school principal. The questions were:

1) Do you swallow the tablets that are given out? (Health worker shows the child the tablets).
2) Have you heard of lymphatic filariasis?
3) Do you know how you get lymphatic filariasis?
   If 'yes', How?
4) Do you use protection against mosquitoes?
   If 'yes', what type?

8.3.4 Statistical Analysis

Data were analysed using Stata (Version 9.0). For continuous data (age) means were compared using t-tests.

For categorical data, logistic regression was used in uni-variable analyses. In the children aged 7 to 10 years, the association between MDA compliance and whether the child had heard of LF (key independent variable) was assessed as well as potential confounding variables including gender, age, use of mosquito protection and number of residents in the household. For the CFA positive group, available data also described socio-economic variables including employment, amount of time spent around the home or away from the home at work, whether their leisure time was spent

predominantly indoors or outdoors, whether they had tested positive for LF in previous years, and, if applicable, if their household was within the identified spatial clusters of infection (Chapter 7).

In multivariable logistic regressions, the effects of potential confounders on the association between MDA compliance and whether the child or adult had heard of LF were assessed by stepwise inclusion of each variable in the model. A potential confounding variable was defined as one which caused a change of +/- 5% in the odds ratio.

Separate analyses were performed to compare areas with high and low Mf prevalence and to compare areas with high and low CFA prevalence.

8.4 RESULTS

8.4.1 CFA Positive Group

8.4.1.1 Uni-variable analyses

Of 153 participants in this group, 69% (n = 105) were from villages in areas with high Mf prevalence and of these the majority (88%) were from villages in areas with high CFA prevalence (Table 8.1a). Comparing areas with high prevalence to areas with low prevalence, for both Mf and CFA, there was no statistically significant difference between the proportions of males and females (P = 0.129 and P = 0.546 respectively). The average age of

participants when defining the groups by high or low Mf prevalence was also similar (35 years and 38 years respectively) (t = 0.74; P = 0.459). However, the average age of participants in the high CFA prevalence group (35 years) was less than the average age in areas with low CFA prevalence (46 years) (t = 2.47; P = 0.015).

Among the 153 CFA positive participants, 67% (n = 103) reported MDA compliance and 56% (n = 86) had heard of LF. In uni-variable analyses, those who reported that they had heard of LF were around three times more likely to report MDA compliance than those who had not heard of LF (P = 0.005; Table 8.2). Furthermore, there was a similarly strong association between self-reported MDA compliance and whether the participant had heard of the national LF programme (P = 0.003; Table 8.2). However, it is of interest that among those who stated that they had not heard of LF, 37% reported they had heard about the national LF programme, while just 14% of those who had heard of LF said they had not heard about the national programme (data not shown).

Of the 86 participants who had heard of LF, 57% (n = 49) said they knew how it is transmitted with most of these (94% = 46/49) reporting they believed it was transmitted by mosquitoes. Although there was no statistically significant association between MDA compliance and self-reported use of any mosquito protection (P = 0.818; Table 8.2), the majority of the sample (79% = 121/153) reported they used some form of mosquito protection and most of these (84% = 102/121) said they used a bednet.

The data show that those who reported MDA compliance tended to be older (39 years compared with 30 years; P = 0.006) and more likely to be farmers or plantation workers compared with those who were unemployed (P = 0.012; Table 8.2). There was no significant association between MDA compliance and spending more time outdoors (P = 0.378; Table 8.2). Testing positive for CFA in previous years was reported by 32 participants. Of these, 53% (n = 17) reported they had heard of LF, 69% (n = 22) had heard about the national programme and 59% (n = 19) reported they initially tested positive during the years from 2005 to 2007. Those previously CFA positive were more likely to report MDA compliance (84% = 27/32; P = 0.026; Table 8.2). The data also appear to indicate that women tended to be less likely than men to report MDA compliance (P = 0.076; Table 8.2). In addition, those who were employed tended to be more likely to report MDA compliance, although the association was not statistically significant (OR = 1.8, 0.9-3.5, P = 0.099; data not shown). Finally, infected individuals who reported being systematically non-compliant were no more likely to reside within the spatial cluster (Chapter 7) than those who participated in MDAs (P = 1.0; Table 8.2).

8.4.1.2 Multi-variable analyses

The results of multiple logistic regressions are tabulated (Table 8.3). To assess the impact on the association between MDA compliance and knowledge of LF, potential confounders were added one at a time sequentially in the regression model. Inclusion of the variables: age, gender,

previous CFA positivity, hours spent at home and hours spent at work each caused changes of +/- 5% in the OR. Although the association weakened, those who reported that they had heard of LF remained significantly more likely to report MDA compliance than those who had not heard of LF (P = 0.034; Table 8.3). The association remains strong when data were analysed for areas with high Mf prevalence (P = 0.042) or CFA prevalence (P = 0.02), but when data for areas of low Mf/CFA prevalence were analysed there was no association (P = 0.56 and P = 0.97 respectively; Table 8.3).

8.4.2 Children Aged 7 to 10 Years

8.4.2.1 Uni-variable analyses

Of the 309 children surveyed, 39% (n = 119) were from villages in areas with high Mf prevalence (Table 8.1b). Comparing areas with high Mf prevalence to areas with low Mf prevalence, there was no statistically significant difference between the proportions of males (54% and 57% respectively) and females (46% and 43% respectively) (P = 0.537). Almost two-thirds (63%) of the 309 children were from villages in areas with high CFA prevalence (Table 8.1b). Areas with high CFA prevalence also had similar proportions of males and females in the sample (57% and 43% respectively) as areas with low CFA prevalence (54% males, 46% females; P = 0.665).

Among the children, 48% (n = 147) reported MDA compliance and 27% (n = 84) had heard of LF. In uni-variable analyses, those who reported

that they had heard of LF were also around three times more likely to report MDA compliance than those who had not heard of LF (P < 0.001; Table 8.4).

Of the 84 children who had heard of LF, 35% (n = 29) said they knew how it is transmitted with the majority (93% = 27/29) reporting they believed it was transmitted by mosquitoes. Unlike the infected group, the association between MDA compliance and self-reported use of any mosquito protection among the children was statistically significant (P < 0.001; Table 8.4). Additionally, the majority of the children (84% = 261/309) reported they used some form of mosquito protection and most said they used a bednet (78% = 203/261).

The data also indicate that those children living in crowded houses with between six and ten residents tended to be significantly less likely to report MDA compliance (P = 0.038; Table 8.4). For example, those children living in houses with six to ten residents were around half as likely as those living in houses with between two and five residents to report MDA compliance. There were no statistically significant differences between those who reported MDA compliance and those who did not in terms of gender and age (P > 0.05; Table 8.4).

8.4.2.2 Multi-variable analyses

To assess their impact on the association between MDA compliance and knowledge of LF, potential confounders were added one at a time

sequentially in multiple logistic regressions (Table 8.5). Only the variable 'use mosquito protection' caused a change of +/- 5% in the OR. In multi-variable analysis with 'use mosquito protection' included as a confounder, those who reported that they had heard of LF remained significantly more likely to report MDA compliance than those who had not heard of LF ($P < 0.001$; Table 8.5). Unlike the data from infected persons, the association is stronger where data for areas with low Mf prevalence are considered alone ($P = 0.003$) and is around the same level, and statistically significant, in areas with both high CFA ($P = 0.003$) and low CFA prevalence ($P = 0.016$) (Table 8.5).

Table 8.1a: Demographics of CFA positive individuals participating in the questionnaire (n = 153). Ongoing transmission was defined as village of residence having either detectable Mf positives (high or low) or ≥ 1% CFA prevalence in children aged ≤ 10 years (WHO, 2005).

Characteristic	Mf high	Mf low	CFA high	CFA low
Male	73	39	97	15
Female	32	9	37	4
Total participants	**105**	**48**	**134**	**19**
Median age, years	36	37	36	47
Age range, years	5-90	2-79	2-90	4-79

Table 8.1b: Demographics of children 7 to 10 years participating in the questionnaire (n = 309). Ongoing transmission was defined as village of residence having either detectable Mf positives (high or low) or ≥ 1% CFA prevalence in children aged ≤ 10 years (WHO, 2005).

Characteristic	Mf high	Mf low	CFA high	CFA low
Male	64	109	111	62
Female	55	81	84	52
Total participants	**119**	**190**	**195**	**114**

Table 8.2: Odds ratios (ORs) and 95%-CI for factors compared with MDA compliance in 153 CFA positive individuals in five Samoan villages as determined with uni-variable regression modelling. Significant associations (P < 0.05) for MDA compliance (key independent variable) are highlighted in **bold**.

Factor	Value	MDA compliance		OR	95%-CI	P*
		No	Yes			
Have you heard of LF?	No	30	37	1.0		
	Yes	20	66	2.7	1.3-5.4	**0.005**
Heard of national programme?	No	26	28	1.0		
	Yes	24	75	2.9	1.4-5.9	**0.003**
Use mosquito protection?	No	11	21	1.0		
	Yes	39	82	1.1	0.5-2.5	0.818
Gender	Male	32	80	1.0		
	Female	18	23	0.5	0.2-1.1	0.076
Age (continuous variable)	Mean	30	39	|t|=2.79		
	SD	19	18			**0.006**
Number of residents in the household	2-5	11	36	1.0		
	6-10	27	48	0.5	0.2-1.2	
	≥ 11	12	19	0.5	0.2-1.3	0.244
Paid employment	Unemployed	26	39	1.0		
	Employed	11	11	0.7	0.3-1.8	
	Farmer/plantation	13	53	2.7	1.2-6.0	**0.007**
Hours spent at home	< 4 hours	27	60	1.0		
	4 hours or more	23	43	0.8	0.4—1.7	0.619
Hours spent at work	Do not work	29	45	1.0		
	< 4 hours	1	13	8.4	1.0-67.5	
	4 hours or more	20	45	0.2	0.7-2.9	**0.033**
Where leisure time	Indoors	28	52	1.0		
	Outdoors	7	20	1.5	0.6-4.1	0.378
CFA previously	No	45	76	1.0		
	Yes	5	27	3.2	1.1-8.9	**0.026**
Part of spatial cluster (Chapter 7)	No	18	36	1.0		
	Yes	15	30	1.0	0.4-2.3	1.000

* Likelihood-ratio chi^2

Table 8.3: Odds ratios (ORs) and 95%-CI for factors compared with MDA compliance in 153 CFA positive individuals in five Samoan villages as determined with multivariable logistic regression modelling. The effects of potential confounders (outlined in Section 8.3.4) on the association between MDA compliance and knowledge of LF were assessed in the sample overall, in analyses restricted to areas where Mf prevalence was high and to where it was low and where CFA prevalence was 'high' and where it was 'low'. Significant associations (P < 0.05) with MDA compliance are highlighted in **bold**.

Factor	Value	N	OR	95%-CI	P*
Sample overall		153	2.4	1.1-5.3	**0.034**
Mf prevalence	High	105	2.9	1.0-7.9	**0.042**
	Low	48	1.6	0.3-7.8	0.560
CFA prevalence	High	134	2.8	1.2-6.6	**0.020**
	Low	19	1.1	0.0-105.7	0.970

* Likelihood-ratio chi^2

Table 8.4: Odds ratios (ORs) and 95%-CI for factors compared with MDA compliance in 309 children (aged 7 to 10 years) in five Samoan villages as determined with uni-variable regression modelling. Significant associations for MDA compliance (key independent variable) are highlighted in **bold**.

Factor	Value	MDA compliance		OR	95%-CI	P*
		No	Yes			
Have you heard of LF?	No	135	90	1.0		
	Yes	27	57	3.2	1.9-5.4	**< 0.001**
Use mosquito protection?	No	39	9	1.0		
	Yes	123	138	4.9	2.3-10.4	**< 0.001**
Gender	Male	92	81	1.0		
	Female	70	66	1.1	0.7-1.7	0.765
Age (continuous variable)	7	36	35	1.0		
	8	39	45	1.2	0.6-2.2	
	9	44	36	0.8	0.4-1.6	
	10	43	31	0.7	0.4-1.4	0.481
Number of residents in household	2-5	26	34	1.0		
	6-10	108	77	0.5	0.3-1.0	
	≥ 11	28	36	1.0	0.5-2.0	**0.038**

* Likelihood-ratio chi^2

Table 8.5: Odds ratios (ORs) and 95%-CI for factors compared with MDA compliance in 309 children (aged 7 to 10 years) in five Samoan villages as determined with multivariable logistic regression modelling. The effects of potential confounders (outlined in Section 8.3.4) on the association between MDA compliance and knowledge of LF were assessed in the sample overall, in analyses restricted to areas where Mf prevalence was high and to where it was low and where CFA prevalence was 'high' and where it was 'low'.

Factor	Value	N	OR	95%-CI	P*
Sample overall		309	3.0	1.7-5.2	**< 0.001**
Mf prevalence	High	119	1.9	0.8-4.7	0.139
	Low	190	4.1	2.0-8.4	**< 0.001**
CFA prevalence	High	195	3.0	1.5-6.3	**0.003**
	Low	114	2.8	1.2-6.6	**0.016**

* Likelihood-ratio chi^2

8.5 DISCUSSION

This exploratory study underlines the link between knowledge and awareness of LF and compliance with treatment campaigns in Samoa. This observation between knowledge of LF and/or national campaigns and MDA compliance is not a new concept for the global LF programme (Rauyajin ***et al.***, 1993; Rauyajin ***et al.***, 1995; Ramaiah ***et al.***, 2000b; Ramaiah ***et al.***, 2001; Yahathugoda ***et al.***, 2003; Babu and Kar, 2004; Mathieu ***et al.***, 2004; Ramaiah ***et al.***, 2006; Gunawardena ***et al.***, 2007; Showkath Ali ***et al.***, 2007; Amarillo ***et al.***, 2008; Babu and Mishra, 2008; Mukhopadhyay ***et al.***, 2008; Showkath ***et al.***, 2008; Talbot ***et al.***, 2008; Malecela ***et al.***, 2009; Cantey ***et al.***, 2010) and, in fact, health education and information has been identified as a crucial accompaniment to MDA campaigns in other public health programmes (MacCormack and Snow, 1986; Marsh ***et al.***, 1996; Guanghan ***et al.***, 2000; Katabarwa ***et al.***, 2000a; Katabarwa ***et al.***, 2000b; Brooker ***et al.***,

2001; Lansdown ***et al.***, 2002; Faulkner ***et al.***, 2003; Mwanga ***et al.***, 2004; Fleming ***et al.***, 2009; Choi ***et al.***, 2010; Lillerud ***et al.***, 2010). Initially, for the LF programme, population compliance was reliant on the high prevalence of chronic cases, serving as an incentive for community participation, and initial comprehensive educational campaigns (Malecela ***et al.***, 2009). As disease prevalence declines, it becomes challenging to convince asymptomatic individuals to consume tablets because of the misconception that only those with visible signs of disease are infected (Wynd ***et al.***, 2007; Bockarie and Molyneux, 2009). As prevalence of LF decreases the need to target these systematically non-compliant individuals escalates. Health education needs to refocus on the effects of disease in those asymptomatic (Wynd ***et al.***, 2007).

In 1999, Samoa was the first country to conduct MDAs under the direction of the WHO (WHO, 2005; Ichimori ***et al.***, 2007b). The reported MDA coverage for the five rounds conducted in Samoa from 1999 to 2003 was 90%, 57%, 68%, 60% and 80% respectively (PacELF, 2005; Huppatz ***et al.***, 2009). It is believed that sustained MDA coverage > 80% annually for four to six years is required in order to interrupt transmission of LF (WHO, 2000; Ismail ***et al.***, 2001; Gyapong ***et al.***, 2005). It could be speculated that since only two of the five rounds achieved this target, low MDA coverage could be a contributing factor to persistent transmission. Additionally, drug distribution for MDA campaigns in the South Pacific did not generally enforce DOT and thus it is unknown whether drugs were consumed which could further affect MDA coverage percentages (Huppatz ***et al.***, 2009). Lack of compliance was

attributed to, in the current exploratory study, a lack of knowledge concerning LF. This highlights a critical niche for the Ministry of Health to design innovative educational campaigns to optimise targeting those non-compliant.

Education campaigns prior to the implementation of each MDA were mandatory for Samoa and were based on materials supplied by PacELF. PacELF designed information, education and communication (IEC) materials for all Pacific countries when it was first initiated (PacELF, 2005). IEC materials included pamphlets, posters, and T shirts which, in 2003, were updated from the dire effects of filariasis morbidity to more positive images of children and adults taking the medication (PacELF, 2005). Interestingly, another means for the community to gain information concerning LF elimination is via the PacELF website and, despite logistical difficulties of internet access, quite a large proportion of those who access the website reside in the Pacific Islands (PacELF, 2005). In Samoa, additional education measures included radio-talk back shows, TV live panels, pamphlets, and calendars demonstrating rugby players swallowing the tablets. Radio-talk back shows allowed individuals to telephone and raise queries concerning the disease and upcoming MDAs. The live TV panel involved discussions between doctors, nurses and health officers with community leaders to raise awareness of the disease and the forthcoming MDA. Since access to technology is limited in areas of Samoa, some isolated residents relied on word of mouth, via relatives or village mayors. The effects of education campaigns on general knowledge within the community were not formally assessed in Samoa using knowledge, attitudes and practice (KAP)

community surveys (WHO Samoa, ***personal communication by Mrs F. Maiava***), which have been shown to significantly improve MDA compliance in Indonesia (Krentel ***et al.***, 2006).

In May 2008, a Communication for Behavioural Impact (COMBI) study was conducted in two villages in Samoa, one with high Mf prevalence and the other with 0% Mf prevalence prior to the June MDA. The purpose of the study was to assess the behaviour of people in their daily lives and how this impacts on their compliance with MDA. The main findings from this study were (WHO, ***personal communication by Mrs F. Maiava***):

(1) Higher rates of compliance could be achieved if drugs were distributed on the weekend;

(2) The maximum impact of awareness of upcoming MDAs was mornings for radio media and evenings for TV media; and,

(3) Higher rates of compliance could be secured if health professionals/personnel distributed drugs rather than people elected from the community.

The success of the high MDA coverage that followed was to some part attributed to the findings of the COMBI study, which were implemented for the June MDA (WHO, ***personal communication by Mrs F. Maiava***). However, since only two villages were assessed because of time constraints, much larger COMBI studies should be conducted in the future. Secondly, I am unaware of the COMBI data including whether the study was randomized or the number of participants. Consequently I cannot draw any conclusions nor

comment on the validity of the findings in the context of compliance with MDAs.

Of concern, in my study in Samoa, only 67% of infected people and 48% of children were recorded as MDA compliant. Consequently, those 33% systematically non-compliant infected individuals remain as reservoirs of infection contributing to the persistent transmission observed in Samoa and, thus, potentially impeding successful elimination of LF (El-Setouhy ***et al.***, 2007; Kyelem ***et al.***, 2008; Huppatz ***et al.***, 2009; Boyd ***et al.***, 2010). Furthermore, although those who had tested CFA positive in previous years were significantly more likely to be compliant, even more alarming is that 16% (n = 5) still admitted to having never participated in MDAs. Whether these individuals, after initially testing positive, were compliant with their own individual treatment programme is unknown.

Those systematically non-compliant infected individuals were no more likely to reside within the spatial cluster than those infected individuals who participated in MDAs. This finding concurs with a recent study undertaken in Haiti, whereby non-compliance was not found to be spatially clustered (Boyd ***et al.***, 2010). The latter study included both CFA positive and CFA negative individuals. It is unknown why there is no observed spatial pattern for non-compliance, especially since infected individuals spatially cluster and are statistically more likely to be non-compliant (Chapter 7) (Boyd ***et al.***, 2010). Studies in Samoa need to include all individuals regardless of infection status to further explore this phenomenon.

The data indicate that the lack of MDA compliance is due to a lack of knowledge concerning the disease since those who had heard of LF were three times more likely to be MDA compliant, which was also the case for children. The link between knowledge and compliance is a crucial finding for the Samoan LF programme, as it highlights the necessity to either implement further educational campaigns or to reassess the existing ones in terms of reaching the target audience appropriately. This is especially highlighted by the discrepancy between people having heard of the disease, but not the national programme to eliminate LF (14%). Furthermore, of those who had tested CFA positive in previous years, only 53% had heard of LF and 69% of the national LF programme. This is very disappointing considering these individuals remembered testing positive, but did not understand the significance. These people require immediate attention. However, further studies must also focus on beliefs, since this can be a strong driving force for drug adherence (Ritter ***et al.***, 2010; Wallerstein and Duran, 2010).

The fact that, for the dataset from infected persons, the link between knowledge of LF and compliance remained strong in areas of high Mf or CFA prevalence in children is encouraging news for the Samoan LF programme. It means that if these areas of residual endemnicity are targeted with appropriate education campaigns and health promotion models, then levels of MDA compliance should increase. Ideally this will decrease prevalence. Why, for the dataset from children, this significant relationship was not observed in areas of high Mf prevalence is unknown. It could be speculated that in areas of high Mf prevalence there is a reduced number of children

attending school because such areas are usually associated with poorer socioeconomic standards (Mwobobia and Mitsui, 1999; Baruah and Rai, 2000; Gunawardena ***et al.***, 2007; Bonfim ***et al.***, 2009). Consequently, children would be less likely to recognise the association between treatment and disease. However, school attendance figures were not confirmed and, thus, the differences in the strength of association between understanding disease and complying with the MDA in different prevalence areas should be further studied.

Individuals within villages with a lower socio-environmental composite index usually represent areas of higher LF endemnicity (Bonfim ***et al.***, 2009). Factors such as overcrowding, poor sanitation, little education, and poor housing construction contribute to a lower composite index (Mwobobia and Mitsui, 1999; Baruah and Rai, 2000; Bonfim ***et al.***, 2009). This is interesting since, in the current study, children from crowded households with a larger number of residents (six to ten people) were about half as likely to be MDA compliant than those children residing in households with lesser than six residents. This raises some important questions regarding why these children are less likely to be compliant.

Overall, the publicity campaigns preceding the annual MDAs in Samoa appeared quite comprehensive involving a range of media. Therefore, in Samoa, efforts must be concentrated on assessing why the message is not being received and why these individuals are systematically non-compliant. Is it because those non-compliant do not have access to media? Is it

because the message itself is not pertinent? Is it because the message is not adequately targeting those non-compliant or young children? Is the compliance status of children related to that of their parents? These questions could be explored using focus group discussions involving the community, trained health workers, and social scientists (Ramaiah ***et al.***, 1996; Awolola ***et al.***, 2000; Katabarwa ***et al.***, 2000a; Lansdown ***et al.***, 2002; Manafa and Isamah, 2002; Mwanga ***et al.***, 2004; Nuwaha ***et al.***, 2004; Wynd ***et al.***, 2007; Massa ***et al.***, 2009). These focus group discussions could also include those individuals identified as systematically non-compliant to discover the reasons behind this. More importantly, beliefs must be explored using the Participatory Action Research (PAR) model (Wallerstein and Duran, 2010) since this can drive drug adherence (western vs. non-western medicine) (Ritter ***et al.***, 2010).

The PAR model aims to bridge the gap between scientists and the community (Wallerstein and Duran, 2010). By doing so, the PAR model increases the likelihood of success by engaging the community and empowering the individuals in the decision-making process (Wallerstein and Duran, 2010). Secondly, the approach aims to identify socioeconomic, cultural, geographic, political, historical, and environmental factors pertinent to the specific public health programme (Wallerstein and Duran, 2010). Lack of information concerning cultural beliefs is a limitation to the current study. Beliefs can be a strong driving force as one woman afflicted with elephantiasis in Vanuatu was ostracised from the village as it was seen as "an act of God" (Hayley Joseph, ***unpublished observation***, 2008). By

including the PAR model in further research higher levels of compliance could be secured. Belief systems and social networking could also potentially contribute to the lack of spatial clustering observed with compliance in the current study. This is because belief systems and social networking have been identified as contributing factors to spatial clustering in non-communicable diseases such as obesity and autism (Chen and Wen, 2010; Liu ***et al.***, 2010).

The data emphasise the necessity for targeting these areas of residual endemic foci with appropriate education campaigns and health promotion models. Specific communication exercises targeted to problem areas has shown improved MDA compliance in Kerala, India (Aswathy ***et al.***, 2009). Secondly, successful elimination of LF in Tanzania has been in part attributed to community acceptance of the prevention and control activities via targeted educational campaigns (Malecela ***et al.***, 2009). In Samoa, new educational campaigns need to address: strategies to motivate participation, innovative public health messages, and different ways to disseminate the health messages. In addition, qualitative research, such as the PAR model, can be conducted to ascertain the reasons behind lack of compliance (Wallerstein and Duran, 2010).

Dissemination of the health message can include the use of church groups, which has had some success in India (Cantey ***et al.***, 2010), schoolchildren (Ekeh and Adeniyi, 1986; Ekeh and Adeniyi, 1988; Marsh ***et al.***, 1996; Shu ***et al.***, 1999; Shu ***et al.***, 2000; Lansdown ***et al.***, 2002; Krentel ***et al.***, 2006;

Gunawardena ***et al.***, 2007; Nandha and Krishnamoorthy, 2007; Malecela ***et al.***, 2009; Massa ***et al.***, 2009), or known members of the community (Katabarwa ***et al.***, 2000b; Gunawardena ***et al.***, 2007). The latter has been identified as unsuitable for Samoa in the recent COMBI survey and, potentially, use of non-medical staff can result in misunderstandings concerning health messages (Cantey ***et al.***, 2010). Secondly, use of church groups may also be inappropriate in areas where there are religious differences, since it has been shown in the OCP that people of differing religious denominations are reluctant to consume tablets distributed by a different church group (Katabarwa ***et al.***, 2000b). In addition, the credibility of the source and the person's status in the community must also be considered. Interestingly, in Samoa, drug distributors consist of health personnel, assisted by women's committees and, in some areas, church groups. Whether the choice of distributor has a direct effect on compliance in Samoa is yet unknown, but requires further investigation.

Another important finding warranting attention is that the older age group who were employed tended to be compliant. Whether this was because of a wider knowledge base, social contact with other co-workers, or a higher level of education is unknown. It has been found previously that awareness of protection against mosquito biting was positively associated with better education and employment of the head of the household (Mwobobia and Mitsui, 1999). Whether this finding is transferable to MDA compliance is unknown, but in Sri Lanka those earning a middle income were found to have higher compliance than the lower income earners (Gunawardena ***et al.***,

2007). The finding from the current study could be exploited to ascertain why employed personnel are more likely to be MDA compliant. There were no significant associations between compliance and whether an individual spent more time indoors or outdoors.

Males tended to be compliant, albeit not at a significant level. This finding is interesting since men in Samoa have a three to five-fold higher Mf prevalence than females (Ichimori ***et al.***, 2007b). Consequently, the higher MDA compliance reported for males is encouraging news for the Samoan LF programme. The higher rate of non-compliance in females also correlated with other studies in Haiti, Sri Lanka and India (Ramaiah ***et al.***, 2000b; Mathieu ***et al.***, 2004; Gunawardena ***et al.***, 2007; Talbot ***et al.***, 2008). It has been speculated that the higher rates of non-compliance in women could be due to previous decisions to not treat women of reproductive age or because the drugs are not distributed to pregnant women (PacELF, 2006; Talbot ***et al.***, 2008). However in Samoa, those women who are not excluded due to the aforementioned reasons do have higher compliance rates than men (WHO, ***personal communication by Mrs F. Maiava***), which is possibly the cause of the higher infection prevalence in the male demographic group (Mahoney and Kessel, 1971; Ichimori ***et al.***, 2007b) (Chapter 6). Other possible reasons for the lower compliance in females observed in the current exploratory study could relate to anecdotal evidence. In the Pacific it was believed that the administered drugs caused sterility in females when the health message was intended to be sterility of female worms. This reiterates the importance of accurate health messages reaching the community in order

to successfully control this disease. It also highlights the necessity to explore the beliefs of the community.

Studies have highlighted schoolchildren as a potential resource of information on public health campaigns for the rest of the community (Ekeh and Adeniyi, 1986; Ekeh and Adeniyi, 1988; Marsh ***et al.***, 1996; Shu ***et al.***, 1999; Shu ***et al.***, 2000; Lansdown ***et al.***, 2002; Krentel ***et al.***, 2006; Gunawardena ***et al.***, 2007; Nandha and Krishnamoorthy, 2007; Malecela ***et al.***, 2009; Massa ***et al.***, 2009). Previous innovative studies have demonstrated the success of using comic books to specifically target children, or handing out pamphlets/leaflets to be passed onto parents (Marsh ***et al.***, 1996; El-Setouhy and Rio, 2003; Malecela ***et al.***, 2009). Parents are more likely to trust either older children (> 12 years) or propaganda coming directly from the school as credible rather than younger children (Marsh ***et al.***, 1996). However, for this strategy to be feasible in Samoa, the following must be determined: 1) community perceptions on using schoolchildren as a source of information, and, 2) levels of school attendance. Furthermore, current levels of knowledge of LF by the children need to be improved. Especially since, in Samoa, only 27% of interviewed children aged 7 to 10 years had heard of LF, which demonstrates the lack of appropriate educational campaigns targeting this demographic group. Targeting a specific cohort could maximise the educational message without straining resources such as using health workers to visit every household in a community.

Additionally, for children, there was a significant association between MDA compliance, knowledge of LF, and the use of mosquito protection. Why is there a strong association between MDA compliance, knowledge, and mosquito protection? Do these young children receive this important message through school education or are their parents/guardians aware of the disease? These important questions need to be answered in order to create an effective community-wide educational campaign. The synergistic relationship observed among these three parameters may be because children who are aware of the disease are more likely to want to protect themselves, either through MDA compliance or mosquito protection. This synergism between mosquito protection and MDA compliance has been identified previously in India (Cantey ***et al.***, 2010). This is encouraging, as it emphasises the need to continue educating the children concerning the dire effects of the disease in order to secure a higher MDA compliance rate.

Both infected individuals and children were more likely to report bednets as their method of protection. In Samoa, the predominant vector is the day-biting ***Ae. polynesiensis*** (Samarawickrema ***et al.***, 1987a; Samarawickrema ***et al.***, 1987b; Samarawickrema ***et al.***, 1993; Ichimori, 2001); thus the use of bednets in these areas is likely to be ineffective (Burkot ***et al.***, 2002). Therefore, the community requires re-education on other ways to protect themselves against mosquito bites such as the use of mosquito coils, the reduction of potential breeding containers in and around their households, or the use of personal repellents (Burkot ***et al.***, 2007; Lambdin ***et al.***, 2008; Mukhopadhyay, 2010). Unfortunately, implementation of other personal

protection measures against mosquitoes results in some personal expense, which might not be feasible for long-term sustainability (Babu ***et al.***, 2007b).

Scheduling further MDA rounds in Samoa to reduce the reservoir of infection will not succeed if these issues concerning non-compliance and the need to strengthen education are not addressed. Unfortunately, inferences cannot be made from the current exploratory study as to whether infected individuals are more likely to be non-compliant, since data were available only for those CFA positive. Therefore, it is imperative to conduct future community surveys in these areas of residual endemnicity to ascertain if non-compliance rates are higher in the infected people. This limitation does not detract from the crucial finding that 33% of infected individuals were systematically non-compliant and this was significantly associated with a lack of knowledge concerning LF. Another potential limitation from this exploratory study is, like any retrospective study, there is a degree of reporting and recall bias (Talbot ***et al.***, 2008; Stoddard ***et al.***, 2009; Cantey ***et al.***, 2010) and, despite reassurance that all responses were valid, there may have been concern from the participants about providing the wrong answers. Secondly, previous studies have demonstrated that the status of the interviewee can potentially affect the respondent's answers (Boyd ***et al.***, 2010). In the current study, an employee of the Ministry of Health volunteered to conduct the interviews. If this impacted upon the answers to the questions, I would have expected most people to have answered "yes" to both MDA compliance and hearing of the disease. Since this was not observed, it was concluded that the status of

the interviewee did not significantly impact the answers to the questionnaire or alter data collection.

Globally there is growing recognition of the need for community involvement and effective health education (Rauyajin ***et al.***, 1993; Rauyajin ***et al.***, 1995; Ramaiah ***et al.***, 2000b; Ramaiah ***et al.***, 2001; Yahathugoda ***et al.***, 2003; Babu and Kar, 2004; Mathieu ***et al.***, 2004; Ramaiah ***et al.***, 2006; Gunawardena ***et al.***, 2007; Showkath Ali ***et al.***, 2007; Amarillo ***et al.***, 2008; Babu and Mishra, 2008; Mukhopadhyay ***et al.***, 2008; Showkath ***et al.***, 2008; Talbot ***et al.***, 2008; Malecela ***et al.***, 2009; Cantey ***et al.***, 2010). Although 30 years ago the need for the introduction of qualitative research into the LF programme was recognised (Nelson, 1981), there appears to be little responsive action. Unfortunately, the move from theory to practice can often be limited because of economic constraints, time, and lack of personnel (Marsh ***et al.***, 1996). However, the investment will benefit the programme in the long run by potentially securing higher rates of compliance. Secondly, if educational campaigns are based on current policies it is difficult to change these behaviours from the country level if policies are not changed at the global WHO level. However, intervention is required immediately if LF is to be successfully eliminated (Talbot ***et al.***, 2008). Health messages should be culturally appropriate and emphasise that all individuals living within endemic areas would benefit from MDA compliance, possibly by reintroducing images of filariasis morbidity (Kumar ***et al.***, 2009; Cantey ***et al.***, 2010). In Sri Lanka, individuals who were aware of the personal benefits of MDA and the severity of progression of disease, even in the absence of clinical symptoms, were

more likely to be compliant (Gunawardena ***et al.***, 2007). Consequently, the lessons learnt from the LF programme could well aid other public health programmes in the future. That is to incorporate social science as a prerequisite from the outset to be proactive and pre-empt possible non-compliance.

The findings identified crucial gaps which could potentially impede successful LF elimination in Samoa. In order to improve MDA compliance, collaboration with social scientists and community members should be a priority to develop pertinent strategies for the health message to reflect local understandings, interpretations of disease, and behaviours. This can be achieved using the PAR model (Wallerstein and Duran, 2010). In order to maximise MDA compliance, it is necessary to re-evaluate the current strategies for dissemination of this information, including the types of media, and exploring other avenues such as school based education. Infected, but non-compliant, individuals and/or schoolchildren represent the prime cohort for targeting these educational messages. How this is achieved, and sustained long-term, requires further investigation, but if LF is to be successfully eliminated in Samoa high population compliance needs to be secured (Molyneux and Zagaria, 2002; Gunawardena ***et al.***, 2007). These preliminary exploratory findings are exciting in terms of aiding successful LF elimination in Samoa and their potential impact on other country programmes.

In summary, the key findings from the research described in this chapter were:-

1) Adults and children were significantly more likely to participate in MDAs if they understood LF and, for children, this extended to the use of mosquito protection;
2) 33% of infected individuals and 48% of children were systematically MDA non-compliant;
3) Of those infected, 16% who had tested positive in previous years admitted to being systematically non-compliant;
4) Females tended to be less compliant than males;
5) Children from overcrowded households were less likely to participate in MDAs; and,
6) Of those that used mosquito protection the majority used a bednet.

Thus it can be concluded that:-

1) New education campaigns, including innovative ways to deliver the health message, must be instigated with collaboration from social scientists to target those systematically non-compliant;
2) These campaigns must include more information on personal protection against mosquitoes;
3) Future work is warranted to ascertain the level of compliance in uninfected individuals;

4) Introduction of the PAR model is required to qualitatively explore beliefs, barriers and solutions in order to expand community involvement in the elimination of LF; and,
5) Without addressing these issues elimination of LF in Samoa will continue to be unsuccessful.

CHAPTER 9

GENERAL DISCUSSION

9.1 DISCUSSION

Public health elimination programmes require diagnostic tools to assess the effectiveness of preventative chemotherapy or other control efforts. For LF elimination, Mf and CFA testing are currently the only diagnostic methods approved by the WHO (WHO, 2005). These diagnostic tools were appropriate whilst prevalence of disease was high. However, as countries reach the end stages of LF control, final epidemiological surveys need to be carried out to identify residual areas of endemnicity or to declare LF eliminated. This stage of the programme is problematic and intense debate is ongoing as to which diagnostic method to use, since Mf and CFA testing may be inadequate (Helmy ***et al.***, 2006; Njenga ***et al.***, 2007a; Weil and Ramzy, 2007; Molyneux, 2009; Pedersen ***et al.***, 2009; Laney ***et al.***, 2010). It has been suggested that measuring exposure, by antibody serology, could be an alternative to measuring levels of infection during this stage of the programme (Weil and Ramzy, 2007; Tisch ***et al.***, 2008; Mladonicky ***et al.***, 2009; Weil ***et al.***, 2010). However, for this to be feasible, a standardised assay must be used, such as the Filariasis CELISA. It was the proposed hypothesis of the current research to either supersede or complement the current diagnostic tools with the Filariasis CELISA.

The Filariasis CELISA will be an asset to the LF elimination programme in this challenging phase of low prevalence. The assay performed comparably well to the research-based Bm14 assay prototype and had higher sensitivity (Weil ***et al.***, 2010). Additionally, the assay was easily adaptable for field testing by using filter paper sampling (Joseph and Melrose, 2010), which is favourable for large surveys. The PPV of 60% for filter paper samples (Joseph and Melrose, 2010) did not adversely affect the results. It must be emphasised that for antibody serology to be useful it must identify problem areas and also confirm the absence of transmission. If false positivity was an issue, we would not have observed a significantly low antibody prevalence rate in Tonga and Vanuatu (< 10%), where LF transmission had ceased (Huppatz ***et al.***, 2009). Furthermore, in areas where transmission was ongoing, significant clustering of exposed children was identified which overlapped spatial clustering of infected individuals. If false positives adversely affected the results there would have been no discernible geographic pattern of antibody exposure. On the contrary, the data suggest that children and adults are being exposed to LF within these geographical limits, with the expectation of a high number of positive children. Consequently, the current format of the Filariasis CELISA is adaptable for filter paper sampling and will reflect the transmission dynamics within the surveyed area.

This innovative research not only demonstrated the favourable advantages of incorporating antibody serology into the available repertoire of diagnostic methods, but also showed for the first time evidence of spatial clustering of

LF in an ***Ae. polynesiensis*** endemic area. In addition, there was evidence of spatial clustering of exposure in conjunction with Mf/CFA positive individuals. These novel findings will have immense impact upon the current WHO guidelines and guide surveillance efforts. Furthermore, the community study in Samoa highlighted the requirement for dynamic educational campaigns to specifically target non-compliant infected individuals and young children born after the initiation of the LF elimination programme under the auspices of the WHO. This could be explored using a PAR model to engage the community (Wallerstein and Duran, 2010).

Prevalence of LF increases with age (Mahoney and Kessel, 1971; Tisch ***et al.***, 2001; Terhell ***et al.***, 2002; Beuria ***et al.***, 2003; Njenga ***et al.***, 2007a; Mladonicky ***et al.***, 2009). This is observed in both high prevalence and low prevalence settings (Chapter 6) (Lammie ***et al.***, 1994; Mladonicky ***et al.***, 2009). Currently, no knowledge exists concerning the relationship between resurgence with low levels of transmission and the time it takes to become CFA positive after exposure. In this research, CFA testing of children alone did not reflect the presence of Mf positive individuals in the community and ongoing transmission of LF. Consequently, in the proposed CTS there are potential inherent problems for surveying CFA positivity alone in children as a means of identifying resurgence. It is estimated that a person needs to be bitten thousands of times by an infective mosquito to produce one new Mf positive case (Hairston and de Meillon, 1968; Southgate, 1984). Thus, acquisition of CFA positivity in childhood remains a product of the transmission intensity (Manguin ***et al.***, 2010) and raises the questions:-

1) If transmission is occurring at a low level, and there is potential for escalation and resurgence, would a child continually exposed to LF become CFA positive by the time they are 5 or 6 years old?
2) If so, would it be a high enough level to be detected by the ICT or Og4C3 ELISA?
3) If they were CFA positive by this age would this mean that transmission had already been ongoing for years, making the implementation of control efforts at this stage more difficult?

Whereas, it is well documented that antibody conversion occurs early during exposure (Eberhard and Lammie, 1991; Hitch ***et al.***, 1991; Gao ***et al.***, 1994; Mahanty ***et al.***, 1994; Bailey ***et al.***, 1995; Lammie ***et al.***, 1998; Weil ***et al.***, 1999) and as few as two infective larvae induce an antibody response in the murine model (Carlow and Philipp, 1987). Consequently, since the outcomes of my research proved the value of antibody serology, the Filariasis CELISA should be incorporated into surveys to detect residual endemnicity and into surveillance after elimination.

A significant impediment to any successful public health programme in developing countries is the necessity for ongoing resources, funding and adequate numbers of personnel. These restrictions could be overcome by using children as an appropriate cohort for surveillance to measure the incidence of cases, which has been shown to be successful in the OCP and is part of the current surveillance strategy for the PICT (Ogunrinade ***et al.***, 1992; Egwang ***et al.***, 1994; Huppatz, 2008). In addition, for sustainability of serological testing in the Pacific, a common testing centre should be

allocated, such as Mataika House in Fiji. In 2007, laboratory staff underwent Filariasis CELISA training supervised by myself. Although the assay has been shown to have little inter-laboratory variation (Weil ***et al.***, 2010), designating Mataika House as the testing centre for PacELF should alleviate any potential problems.

Finally, ongoing transmission in Samoa is a problem. Without making changes to current control policies LF will continue to be a public health problem. Fortunately, the identified spatial clustering of LF will aid control efforts by the potential incorporation of vector control and targeting treatment. Furthermore, educational campaigns can be revamped to target those systematically non-compliant, further reducing the reservoir of infection. Collectively, the findings from my research will aid these changes.

9.2 FUTURE DIRECTIONS AND CONCLUSIONS

The intention is to expand this research to answer the following important questions:

(1) Does spatial clustering of exposure occur in other LF endemic areas?

(2) What mathematical models would be applicable to antibody serology in order to predict transmission thresholds?

(3) Current mathematical modelling is based on transmission with ***Culex*** sp. (Stolk ***et al.***, 2006). What are the thresholds in the highly efficient ***Ae. polynesiensis*** endemic areas?

(4) Is there spatial clustering of LF in another ***Ae. polynesiensis*** endemic PICT such as French Polynesia?

(5) Will the Filariasis CELISA be a feasible diagnostic method in other low prevalence settings globally?

(6) In the study villages in Samoa:-

   a. Are there any geographical areas of interest such as water bodies?

   b. Is there a difference between wet and dry seasons as evidenced in American Samoa? (Lambdin ***et al.***, 2009)

   c. Is there a spatial relationship between breeding habitats and LF cases?

   d. Is there a high non-compliance rate amongst uninfected individuals?

   e. What factors contribute to compliance in young children and how do they gain access to knowledge concerning LF? Could education campaigns be introduced into the school curriculum? Will a PAR approach increase compliance?

The research findings from Samoa will help guide decisions made by the Ministry of Health concerning future MDA campaigns and control efforts. The fundamental need for ongoing and innovative educational campaigns should be explored. Most importantly, this research opens up promising avenues to incorporate vector control, which has been identified as a necessity for successful LF elimination (Kyelem ***et al.***, 2008) and, prior to this research, was thought to be ineffective in ***Ae. polynesiensis*** endemic areas due to the

believed lack of spatial clustering (Bockarie ***et al.***, 2009a; Mladonicky ***et al.***, 2009). Furthermore, as a result of the identified spatial clustering in Samoa, current treatment efforts could be reassessed. Currently, identification of ongoing transmission in Samoa requires the implementation of countrywide MDAs. On the contrary, the current research demonstrates the potential for targeting treatment to only those areas with residual endemnicity and that the radius of targeted treatment will depend on the prevalence of LF. This targeted approach would be beneficial for the Ministry of Health by reducing the resources required. In summary, the combination of introducing vector control, targeted treatment to residual areas of foci as identified by measuring antibody serology, and innovative educational campaigns to target non-compliers should aid successful elimination of LF in Samoa.

Collectively, the data provide crucial information to the LF programme managers in Samoa, and indeed on a global level, concerning the potential usefulness of incorporating antibody serology as a means for identifying residual endemnicity and future surveillance. The promising results from implementing the Filariasis CELISA to detect antibodies should be further investigated on a global level. Further GPS/GIS mapping for serology would be necessary in other epidemiological settings, which has been effectively used in both the OCP and loasis control programme (Baker ***et al.***, 2010). In conclusion, the Filariasis CELISA would be an asset to the LF programme for defining residual endemnicity and during surveillance using a cohort of children. Most importantly, the novel finding of spatial clustering in an ***Ae. polynesiensis*** endemic area will challenge the current dogma for control

efforts in these areas. Furthermore, this research will drive future investigations globally to explore the use of antibody serology in other low prevalence settings and spatial clustering in other ***Ae. polynesiensis*** endemic areas.

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APPENDIX 1

PRODUCT INSERT: THE FILARIASIS CELISA

Bm 14 Lymphatic Filariasis Antibody CELISA (KF3)
Indirect ELISA for the detection of Bm14 specific IgG_4 Antibody
5 Plate Kit

Bancroftian filariasis (LF), caused by the filarial worm, ***Wuchereria bancrofti***, and transmitted through a mosquito vector, affects more than 100 million people in more than 83 countries worldwide[1]. A further billion people are at risk from the infection, which in its chronic stage (elephantiasis) has profound physical, social, and economic consequences, and represents the world's fourth leading cause of permanent disability. The disease has been targeted by the WHO for eradication by the year 2020 through an international programme of mass drug administration (MDA)[2] operated by individual countries in collaboration with the Global Alliance to Eliminate Lymphatic Filariasis (GAELF). To monitor the effectiveness of the LF eradication programme a number of diagnostic tools have been employed, including microfilaremia quantitation and parasite antigen immunoassays, both of the rapid and ELISA format. Specific IgG_4 anti – filarial antibodies have also been proposed as an effective marker for interruption of LF transmission, particularly in young children[3]. The BM14 Filariasis Antibody Kit is an indirect ELISA for the detection of human IgG_4 antibodies to the filarial recombinant antigen, BM14[4,5,6]. Added sensitivity and specificity result from the use of a monoclonal anti – IgG_4 indicator. The test is designed to be used with serum samples as well as blood spot eluates collected on filter paper in the field.

Overview of Filariasis IgG_4 Antibody assay

1. Bring assay components to room temperature.
2. Test samples @ 1:100 dilution, using the sample diluent provided
3. Incubate samples for 2 h @37^{o}C in humidified chamber
4. Wash 4X with PBST
5. Add anti-human IgG_4-HRP conjugate, 45 min incubation @37^{o}C.
6. Wash 4X with PBST

7. Develop wells with TMB substrate for 15 min @ RT

8. Stop reaction by adding stopping solution to each well. Read absorbance values of all wells at dual wavelength (450/620 nm or 450/650 nm).

Kit contents:

- **5 ELISA plates coated with r-Bm14 antigen** (FAMW), sealed dry in aluminium foil bags.
- **1x 250mL PBS/Tween (20x) Wash Buffer concentrate** (FAPT)
- **1x 60mLof Sample diluent (10x)** (FASD)
- **1x 60mL Conjugate diluent** (FACD)
- **1x 0.6mL Peroxidase Enzyme Conjugate (100x) Monoclonal anti-human IgG_4-HRP** (FAPO)
- **1x 60mL Substrate buffer** (FASB)
- **3.0mL TMB Substrate chromogen (20x)** (FASC)
- **2x 30mL of Stop solution** (FASS)

Important Notes:

- Store the kit components at 2-8^{o}C when not in use. Unused strips must be adequately re-sealed with silica gel sachets in the aluminium foil packaging.

- Bring all assay components to room temperature before starting the assay to ensure optimal CELISA performance.

- The wash buffer concentrate (PBS/Tween 20x) will crystallize on storage at 2-8^{o}C. It is critical that crystals are dissolved before dilution. This can be achieved by stirring the solution in a 37^{o}C water bath. Use distilled water to dilute the wash buffer concentrate. **Do not use deionised or ultra-pure MilliQ water as hydrocarbons in these highly processed waters inhibit peroxidase enzymes and may affect the assay**.

Preparation of assay solutions

Working wash buffer using a 20x PBS/Tween concentrate: Prepare a working wash buffer (1x) by diluting the 20x concentrate in distilled water. Mix the wash buffer thoroughly before use.
Sample Diluent 10x concentrate: Prepare the working Sample Diluent (1x) by diluting the 10x concentrate in distilled water. Mix the buffer thoroughly before using to dilute the test samples.
Sample (analyte) dilution: Designate the negative sample as the **Ref-ve** and the positive sample as the **Ref+ve**. Dilute the samples at 1:100 using the working Sample Diluent (1x) buffer. Make sure that the samples are accurately diluted by using a minimum of 5µL of sample into 495µL working sample diluent. **Never dilute the sample by directly mixing 1µL of neat sample into 99 µL sample diluent buffer.**

Sample preparation and plasma elution method: Both serum samples and blood spot eluates can be used for antibody testing. Serum samples should be prepared according to standard procedures. For blood spots, each filter paper circle used in the MDA study normally absorbs ~10 µL blood. Upon drying at room temperature, the spotted blood circles should be stored at -20C in a freezer until used for testing. Prior to use, cut off one circle and drop into a small tube containing 500 µL of 1x sample buffer. Vortex the tube to wet the circle and make sure that the filter paper circle is completely immersed within the sample buffer. Elute the sample overnight by storing the tubes in a refrigerator. The following day, take out the tube and re-vortex the contents well to ensure complete elution of blood from each circle. The resultant eluate is equivalent to a 1:100 serum dilution and can be used in the CELISA assay without further dilution.

Assay Steps

1. Bring all assay components to room temperature.

2. Open the aluminium foil bag containing the antigen coated plate and take out the required number of wells. Include the control samples, Ref-ve, Ref+ve and the blank wells.

3. Dilute the serum samples at 1:100 using the working sample diluent. [**Note**: Test the eluted samples from the filter paper circles straight away without further dilution]. Follow the recommended dilution method as described under the Preparation of assay solutions above.

4. Transfer 100µL of diluted sample into each well and incubate for 120 min at 37^{o}C in a humidified chamber.

Five minutes before the end of the incubation, prepare the working conjugate (1x) by diluting the conjugate concentrate 1 in 100 using the conjugate diluent. Mix thoroughly before use. Protect the diluted conjugate from light

5. Wash the reaction wells with the wash buffer 4 times preferably using an automatic plate/strip washer. Each wash step consists of emptying

the wells followed by refilling with wash buffer. Shake out well contents at end of final wash.

6. Add 100 µL of diluted conjugate to each well.

7. Cover the strips and incubate for 45 min at 37C.

Ten minutes before the end of the incubation, prepare the working substrate solution (1x) by diluting the 20x concentrate in substrate buffer. Allow 1 ml of diluted substrate for a strip of 8 wells.

8. Repeat washing as in step 5.

9. Add 100 µL of substrate solution to each well.

10. Cover the strip and develop in the dark at room temperature. Tap the plate at 5 min intervals for colour development.

11. Develop the blue colour for 15 min.

12. Add 100 µL of stopping solution to each well and mix thoroughly. Addition of stopping solution will turn blue reactive wells yellow. Read the absorbance values immediately at 450 nm or 450/620 nm or 450/650 nm (or 450/750 nm) depending on the availability of filter systems in the plate reader.

Reading and Interpretation of Test Results It is more accurate to take the readings of all reactant wells without blanking the plate reader. The actual absorbance reading of each well is very important in assessing whether the assay has worked or not. By comparing the absorbance of a blank sample, a negative sample and a positive sample, the real reactivity of unknown or test samples can be determined. These readings allow the cut off value (essentially based on mean+3SD of proven negative samples) to be accurately determined. The real reading of unknown or test samples can be calculated based on the differential absorbance of unknown versus proven negative samples. The provisional cut off is determined as the mean plus 3 standard deviations of a panel of known negative samples. Most laboratories have found this absorbance value to be between ~0.2 and 0.4 OD units. Any test sample reacting above this cut off level is considered to be positive.

Overall interpretation $OD_{450/620\ nm}$
Any sample showing OD < 0.2 = definitively negative
Any sample showing OD > 0.4 = definitively positive
Absorbance between OD = 0.2 to 0.4 in determinate sample (Should be re-tested)

Note: A pilot study conducted in-house with 50 blood spot eluates from Australian residents without prior exposure to LF gave a cut-off of 0.25 OD units (mean +3SD).

References:

1. Weil, G.J. and Ramzy, R.M. (2007) Diagnostic tools for filariasis elimination programs. ***Trends in Parasitology* 23 (2)**: 78-82

2. Ramzy, R.M., El Setouhy, M., Helmy, H., Ahmed, E.S., Abd Elaziz, K.M., Farid, H.A., Shannon, W.D. and Weil, G.J. (2006) Effect of yearly mass drug administration with diethylcarbamazine and albendazole on Bancroftian filariasis in Egypt: a comprehensive assessment. ***Lancet* 367 (9515)**: 992-9

3. Ottesen, E.A., Skvaril, F., Tripathy, S.P., Poindexter, R.W. and Hussain, R. (1985) Prominence of IgG_4 in the IgG antibody response to human filariasis. ***Journal of Immunology* 134 (4)**: 2707-12

4. Chandrashekar, R., Curtis, K.C., Ramzy, R.M., Liftis, F., Li, B.W. and Weil, G.J. (1994) Molecular cloning of ***Brugia malayi*** antigens for diagnosis of lymphatic filariasis. ***Molecular and Biochemical Parasitology* 64 (2)**: 261-71

5. Ramzy, R.M., Helmy, H., Faris, R., Gad, A.M., Chandrashekar, R. and Weil, G.J. (1995) Evaluation of a recombinant antigen-based antibody assay for diagnosis of Bancroftian filariasis in Egypt. ***Annals of Tropical Medicine and Parasitology* 89 (4)**: 443-6

6. Lammie, P.J., Weil, G., Noordin, R., Kaliraj, P., Steel, C., Goodman, D., Lakshmikanthan, V.B. and Ottesen, E. (2004) Recombinant antigen-based antibody assays for the diagnosis and surveillance of lymphatic filariasis - a multicenter trial. ***Filaria Journal* 3 (1)**: 9

APPENDIX 2

PRODUCT INSERT: THE OG4C3 ELISA

ELUTION of BLOOD SPOTS for use in the Trop-Ag *W. bancrofti* antigen detection ELISA kit Cat. No. 03-010-05

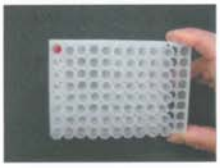
Elution Rack + tubes

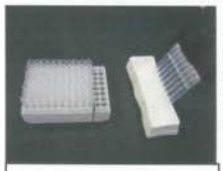
1 Elution tubes in Racks (modified)
Racks of 1 ml tubes designed in a 96-tube (8 x 12) format compatible with the 96-well ELISA plate. The racks are modified: the lower corners have been ground or sliced off to allow complete circulation of water when immersed in a boiling water bath.
The racks are filled with sufficient tubes for up to 80 samples per rack. (The last two columns need not contain tubes as these columns are reserved for the standard antigens.)

Rack / corner off

Add blood spots

2 THREE filter paper protrusions (blood spots) are cut from the central body of the disk with a pair of scissors. Place three blood spots from each patient into a single tube, ie one tube for each patient. To allow the filter paper to reach the bottom of the tube, each blood spot should first be cut into 2 or 3 pieces. Using a small funnel allows the filter paper to be easily added to the tubes.
The three remaining blood spots on the disk can be stored for later use if necessary.





Add Sample Diluent

3 ADD 200 µl of Sample Diluent to each tube. Using a multistepper pipette or similar is an efficient method. Ensure that the pieces of filter paper are fully immersed in the diluent. Samples are best left to elute overnight at 4°C.

Boil Samples

4 BOIL the tubes in a 100°C water bath for five minutes. An electric frypan is ideal for boiling the samples.

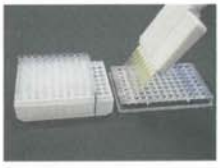
5 CENTRIFUGE the samples at 10,000 *g* for five minutes or 1,000 *g* for 20 minutes using a swing-bucket rotor designed for plates. The clear supernatant fluid contains the stable antigen.

Centrifuge Samples

Transfer to ELISA plate

6 TRANSFER 50 µl of supernatant to a well of an ELISA plate from the Trop-Ag *W. bancrofti* antigen detection kit. Add the standard antigens (these are not diluted or boiled) to the last 2 columns.

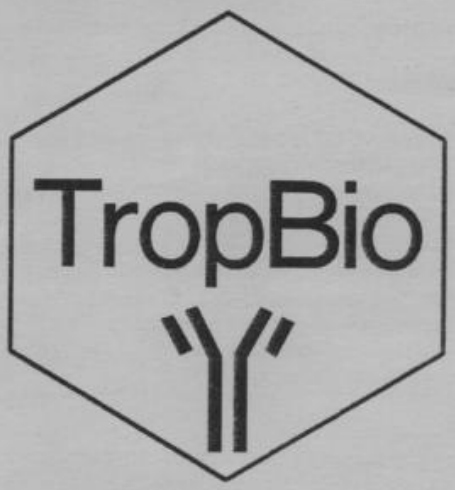
7 PROCEED with the ELISA



Trop-Ag *W. Bancrofti*

Filter Paper Compatible

Five plate kit



ELISA KIT FOR DETECTING AND QUANTIFYING *Wuchereria bancrofti* ANTIGEN

480 test Kit

Catalogue No 03-010-05

Lymphatic filariasis

An antigen detection assay has been developed by **TropBio** and James Cook University of Nor Queensland for the detection of *Wuchereria bancrofti* (Bancroftian filariasis) infection in man. This ass has been marketed to fulfil a perceived need in the testing of human patients suspected of being infect with this parasite.

Lymphatic filariasis in man is caused by infection with the filarial parasites W. *bancrofti and Brugia sp* These parasites inhabit the lymphatics and cause disease by obstruction and secondary inflammato changes to lymph vessels. The disease is transmitted by mosquitoes which ingest microfilariae duri feeding on an infected host and transmit the infective larvae to other individuals at a subsequent feedi

Throughout the world, more than 90 million people are affected by lymphatic filariasis. Most live in t humid tropics in areas such as Africa (south of the Sahara), Egypt, the Indian subcontinent, South-Ea Asia, China, Madagascar, Papua New Guinea, the Pacific Islands, the Philippines and Central and Sou America (World Health Organisation, 1984).

Acute symptoms of lymphatic filariasis primarily involve lymphadenitis and lymphangitis. Recurring fev and pain of affected lymph nodes are the normal sequelae. In some patients, symptoms may be less specifi with fever and malaise being the only symptoms. Some infected individuals are asymptomatic.

Lymphoedema becomes apparent after repeated episodes of lymphadenitis, and swelling of the limbs scrotum may occur. Lymphoedema and elephantiasis may affect the leg, arm and scrotum and occasional the vulva and breasts, but they are usually restricted to the leg below the knee.

With brugian filariasis, more severe inflammatory changes are noted in the lymphatics, whereas bancrofti filariasis has a more extensive swelling of the entire limb.

Filariasis in previously unexposed migrants to an endemic area has a similar clinical course, but can b manifested earlier (6 - 8 weeks) than the normal 7 - 8 month clinical incubation period. Microfilaraem is an uncommon finding in these migrant individuals, reflecting an intense immunological reaction to th parasite (Partono *et al*., 1977).

References

More, S.J., and Copeman, D.B. (1990) A highly specific and sensitive monoclonal antibody-based ELIS for the detection of circulating antigen in bancroftian filariasis. *Tropical Medicine and Parasitolog* **41:** 403- 406

Partono, F., Oernijati, S. and Hudojo, I. (1977) Malayan filariasis in Central Sulawesi (Celebes), Indonesi *Southeast Asian Journal of Tropical Medicine and Public Health* **8:** 452-458

Turner P., Copeman B., Gerisi, D. and Speare R (1992) A comparison of the OG4C3 antigen captur ELISA, the Knott test, an IgG_4 assay and clinical signs, in the diagnosis of Bancroftian filariasis. *Tropica Medicine and Parasitology* **44:** 45-48

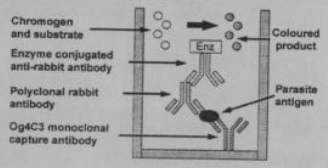
World Health Organisation (1984) Lymphatic Filariasis. *Fourth Report of the WHO Expert Committee o Filariasis*, Geneva. WHO Technical Services 702

Notes on the assay

This kit has been designed to be used in conjunction with the filter paper collection kits (05-001-08 and 05-001-07). Boiling of the filter papers is likely to leach endogenous peroxidase from the erythrocytes in the filter paper. The assay incorporates an additional blocking step to remove this problem and prevent high non-specific background reactions from occurring.

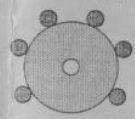
The 96 well microtitre plates supplied are coated with a monoclonal antibody(Og4C3) which has been shown to specifically recognise only *Wuchereria bancrofti* antigen in human sera. Og4C3 will not cross react with human sera infected with *Onchocerca volvulus, Brugia malayi, Brugia timori, Loa loa, Mansonella perstans, Strongyloides stercoralis, Dracunculus medinensis* or *Ascaris lumbricoides*. The cattle parasite *Onchocerca gibsoni* is recognised by this monoclonal antibody and is used to standardise the ELISA.

ELISA configuration (antigen detection) for *W. bancrofti*



The indicator antibody is produced by vaccinating rabbits with purified *Onchocerca gibsoni* antigen. Finally the rabbit globulin is detected by goat anti-rabbit globulin conjugated to horseradish peroxidase. The substrates solution which contains the chromogen ABTS produces a green colour which has maximum absorption between 405 and 414 nm.

Collection of blood on TropBio filter paper disks



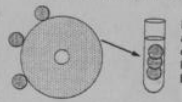
Sample collection protrusions are saturated with blood, dried and transported to the laboratory

Samples for this assay are collected on filter paper disks. One disk is collected per patient. A number can be recorded on the body of the disk prior to collection. Patient details are linked to the record number. A data sheet is supplied as part of the collection kit.

All six protrusions are saturated with blood and the filter paper is thoroughly dried on the rack provided. Each protrusion contains the equivalent of 5 µL of serum. The kit contains small plastic bags punched to allow air movement. These bags are then placed in groups of eighty and placed in a larger press seal bag into which is placed a silica gel sachet to further dry the samples.

The samples are transported to the laboratory. Eight samples will be placed in one column of the plate. The collection kit encourages the arrangement of the samples in blocks of eight which are then arranged in groups of 80 which is sufficient for one test plate. Five test plates containing 400 samples can be assayed in one kit.

Elution of parasite antigen from TropBio filter paper disks



Parasite antigen is eluted by boiling in the EDTA diluent

Three protrusions can be cut from the filter paper disk and placed in the elution tubes. To allow the disks to reach the bottom of the tubes provided in the kit (005-001-08) they should first be cut in half. Diluent containing EDTA is added to the tubes. Three disks will contain antigen equal to 15 µL of serum and this is eluted in 200 µL of diluent. This is equivalent to a 1:13 dilution of a serum sample.

In the kit which uses serum as a sample the dilution is 1:4. The filter paper results are therefore lower than the corresponding serum results. However, as most infected patients have relatively high antigen titres the clinical sensitivity remains high.

Storage

TropBio issues a 6 month Expiry Date where kits can be stored at 2 - 8 °C on arrival. The kits are shipped with some buffers frozen to -80°C to keep the kits cool during shipping. These buffers are likely to be thawed by arrival, but refreezing is unnecessary. However, where the bulk of the kit reagents can be stored at 2-4°C, with the HRPO conjugate (the least stable component) stored at -20°C or even -80°C, the shelf life can be confidently extended to 12 months.

ELISA KIT FOR DETECTING AND QUANTIFYING *Wuchereria bancrofti* ANTIGEN

Filter paper compatible

03-010-05

Manufactured by TropBio Pty Ltd
James Cook University, Townsville
Queensland, Australia 4811
ABN: 97 051 617 424

This kit contains sufficient reagents for 480 tests.

CONTENTS

1. **Microtitre Plates** (in Silver foil pouches)
   Five U-bottom PS microtitre plates pre-coated with Og4C3 monoclonal antibody.
2. **Sample diluent** (Clear solution)
   One 120 mL bottle of diluent at working strength for elution of samples prior to boiling.
3. **Antibody and conjugate diluent**
   (Blue solution) One 100 mL bottle.
4. **Hydrogen Peroxide** (Amber with green cap)
   One x 2 mL tube of H_2O_2 Concentrate.
5. **Standard antigens (1-7)** (Orange cap)
   Seven dilutions of *Onchocerca gibsoni* antigen. Each tube contains 800 µL of standard.
6. **Rabbit anti-*onchocerca* antibody**
   (Yellow cap - Indicator antibody)
   One tube containing 350 µL.
7. **Conjugate** (Purple cap)
   One tube containing 350 µL Anti-rabbit HRPO conjugate.
8. **ABTS Chromogen** (Amber bottle)
   One 60 mL bottle of single component ABTS ready to use.
9. **Washing buffer** (Twin-neck bottle)
   One 250 mL x20 concentrate.
10. **Wash bottle** (optional)

METHOD

All steps carried out at room temperature.

Ensure that all reagents and the microtitre plates are at room temperature before use.

Quantities indicated below refer to those required for the use of **ONE** plate.

To prepare washing buffer, dispense one measure from the x20 dispensing bottle into 500 mL of distilled water.

1. **Preparation and addition of test samples.**

   Cut three protrusions from the filter paper disk and add them to a suitable tube. If the racked tubes supplied in the kit (05-001-08) are used, the protrusions should first be cut in half to allow the disks to reach the bottom of the tubes.

   Add 200 µL of sample diluent to each tube. Leave the blood samples to elute overnight at 4°C. Next morning, place the racked tubes into a 100°C water bath for five minutes.

   After heating in the water bath, centrifuge the samples at 2,000 *g* for 15 minutes. The supernatant contains the heat stable antigen.

   Add 50 µL of supernatant to a test well. Up to 80 samples can be tested per plate using the recommended plate layout diagram.

2. **Adding Standard Antigens and Conjugate Control to columns 11 and 12**

   Following the plate diagram shown opposite, add Standards (1-7, Orange caps) at 50 µL per well in duplicate.
   Do not dilute or boil.
   For conjugate control (CC), add 50 µL of sample diluent (100 mL bottle, clear solution) to wells A11 and A12.

3. **Incubation of Test Samples**

   Place the plate in a humid container and incubate for at least 1.5 hours at room temperature. Plates may be incubated overnight to increase sensitivity.

4. Wash the plate three times with wash buffer, inverting and tapping gently between each wash to remove residual droplets.

5. **Blocking of Endogenous Peroxidase with hydrogen peroxide.**

   Prepare the blocking solution by adding 200 µL of H_2O_2 concentrate to 6 mL of diluted wash buffer. (This treatment is necessary to reduce the interference of endogenous peroxidase released by the haemolysis of red blood cells on the filter paper.)

   Add 50 µL of the diluted blocking solution to all test wells and incubate for 10 minutes at room temperature. (It is not necessary to add the blocking solution to the Standards or Conjugate Controls.)

6. Wash the plate three times as before.

7. **Addition of Rabbit anti-*Onchocerca* Antibody**

   Dilute by adding 50 µL of rabbit anti-*Onchocerca* antibody (Yellow cap) to 6 mL of antibody diluent (Blue solution).

   Add 50 µL of diluted rabbit antibody to all wells and incubate for one hour.

8. Wash the plate three times as before.

9. **Addition of Anti-rabbit HRPO**

   Dilute by adding 50 µL of Anti-rabbit HRPO conjugate (Purple cap) to 6 mL of antibody diluent. Add 50 µL of diluted conjugate to all wells and incubate one hour.

10. Wash the plate three times as before.

11. **Addition of ABTS chromagen**

    Add 100 µL of ABTS (**do not dilute**) to each well and incubate for one hour.

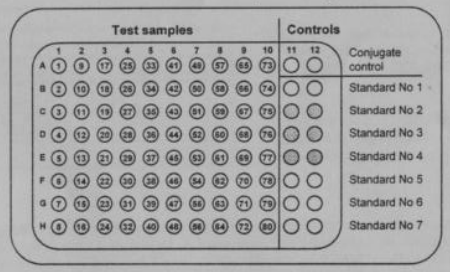
12. **To Read the Reaction**

    Plates can be read with a spectrophotometer at a wavelength between 405 - 414 nm or dual wavelengths of 405-414 and 492 nm.

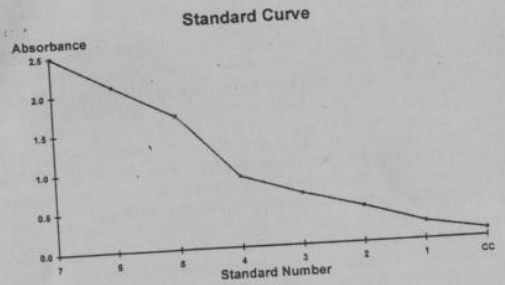
    If necessary, blank the plate reader on wells containing conjugate control or a row of wells containing substrate in a separate blanking plate.

    This kit is to be used for *in vitro* testing purposes only. All components must be disposed of by autoclaving at the completion of the testing.

Standard ELISA plate layout



A typical standard curve for the seven standard antigens is shown below. These values are used to allocate the test samples into one of eight titre groups according to the table shown above.



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Expiry Date

INTERPRETATION OF RESULTS

If the optical density for the high titre control is less than 1.1 or the optical density for the negative control (Standard No 1) is more than 0.3 the test should be regarded as unreliable and should be repeated.

Please note that there can be quite large differences between plate readers. The result will also be influenced by the choice of filter (a 414 nm filter will produce the highest results). The absorbance indicated by the plate reader may also change as the filters deteriorate with age.

Seven control samples are used in duplicate on all plates. These samples are produced using parasite antigen extracted from *Onchocerca gibsoni* nodules. Control sample No 1 contains no parasite antigen. Control samples No 2 to No 7 all contain parasite antigen.

Sample No 2 is at the limit of the sensitivity of the assay. However, it will consistently produce an absorbance higher than the control sample No 1. Very few serum samples will react with a higher absorbance than control sample No 7.

Using these seven control samples it is possible to allocate the test samples into eight titre groups according to the following table. The titre groups are very useful for population studies. Moore and Copeman (1990) allocated antigen units to the seven controls.

Allocation of samples to titre groups

Titre group	Absorbance	Standard No	Antigen units
1	< Control sample No 1	1	<10
2	≤ Control sample No 2	2	32
3	≤ Control sample No 3	3	128
4	≤ Control sample No 4	4	512
5	≤ Control sample No 5	5	2,048
6	≤ Control sample No 6	6	8,192
7	≤ Control sample No 7	7	32,000
8	> Control sample No 7		

A total of 100 filter paper samples from uninfected patients from the Townsville region reacted with the following distribution. Mean OD = 0.149. Standard deviation = 0.045.

100 Australian samples

Titre group	Number of samples
1	83
2	17
3 or more	0

The test samples allocated to titre groups 1 and 2 can be considered to be non-reactors (negative). Samples allocated to titre groups 4 to 8 can be considered to be reactors (positive).

Samples allocated to titre group 3 can be considered to be equivocal or suspect reactors. In a recent study in New Guinea, Ghana, the Philippines and India this group represented up to 10% of the test samples. None of the Australian samples from an uninfected population were allocated to group 3. It is very likely that samples allocated to group 3 are reacting in the assay. Further data will be collected on this group to determine their status.

APPENDIX 3

HUMAN ETHICS APPROVAL H1423

ADMINISTRATIVE DOCUMENTATION HAS BEEN REMOVED

ADMINISTRATIVE DOCUMENTATION HAS BEEN REMOVED

APPENDIX 4

HUMAN ETHICS APPROVAL H2816

ADMINISTRATIVE DOCUMENTATION HAS BEEN REMOVED

ADMINISTRATIVE DOCUMENTATION HAS BEEN REMOVED

APPENDIX 5

TITRATION OF THE PNG SERA SAMPLES

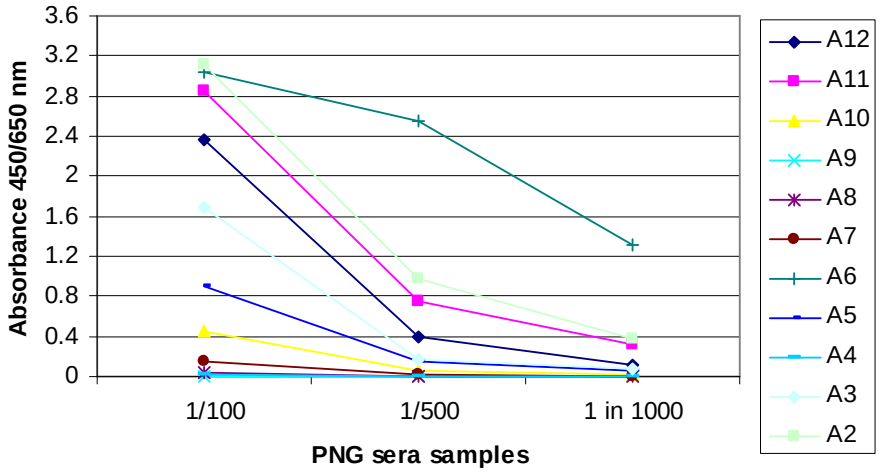

Figure A6.1: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

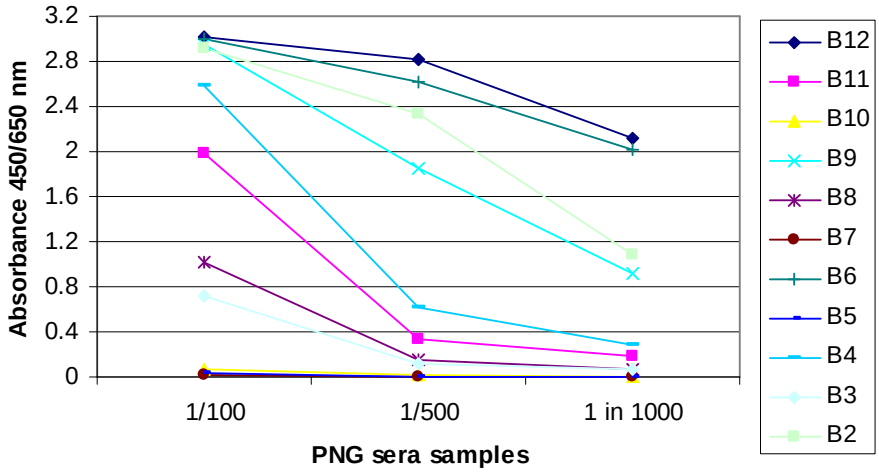

Figure A6.2: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

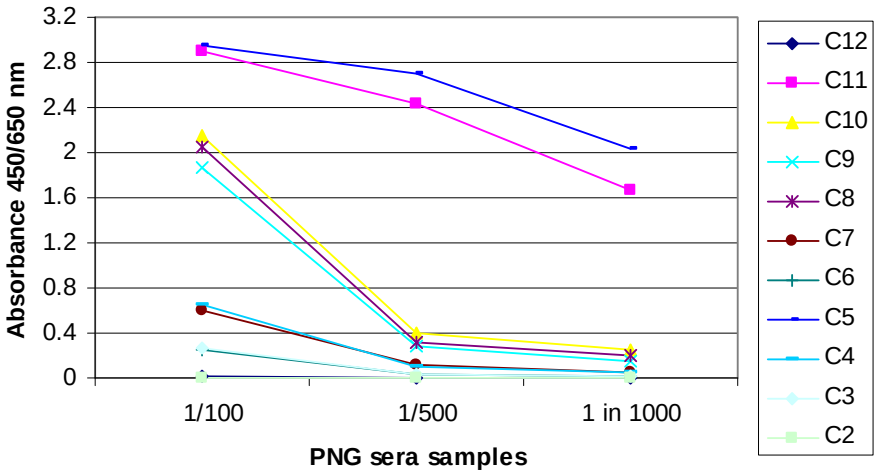

Figure A6.3: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

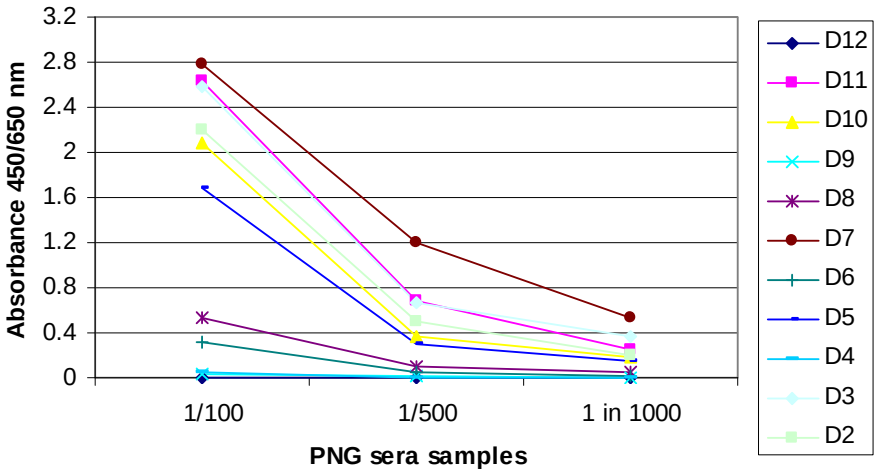

Figure A6.4: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

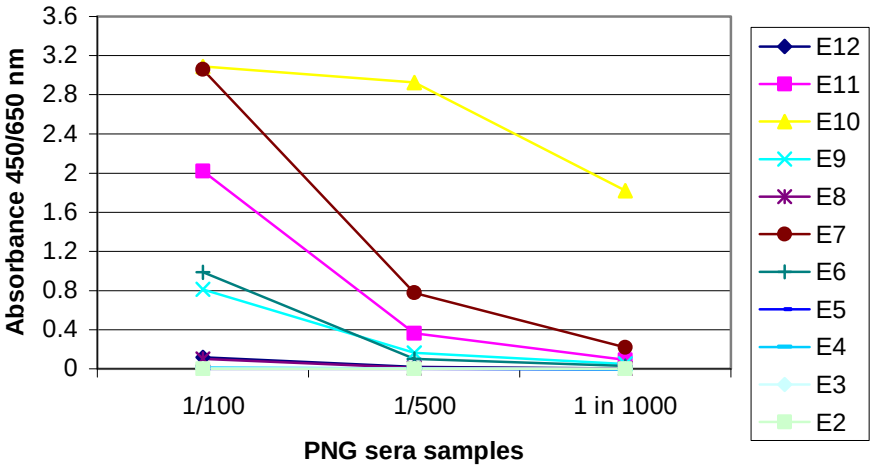

Figure A6.5: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

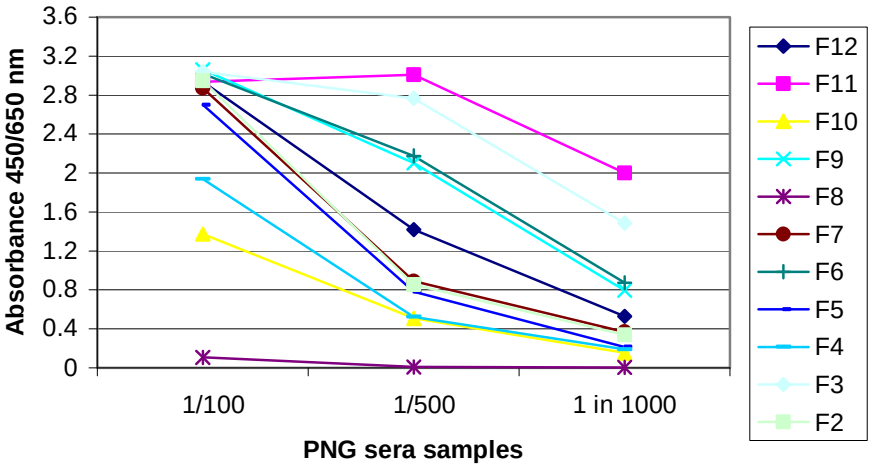

Figure A6.6: OD absorbance values for 11 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

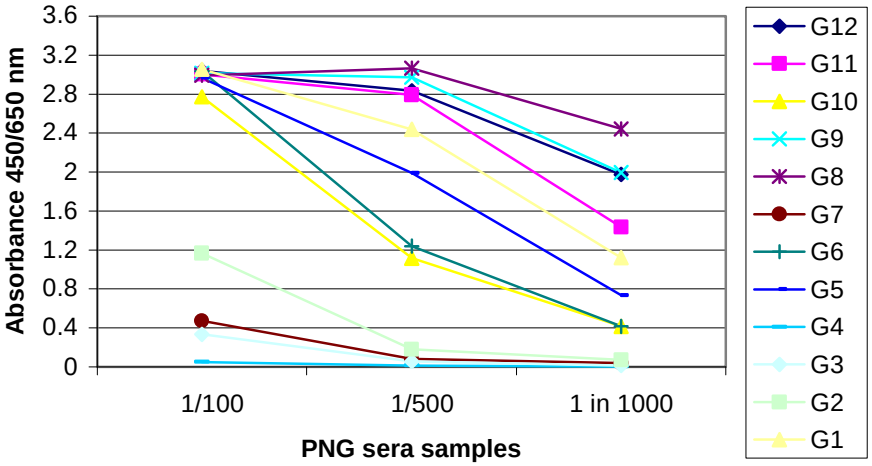

Figure A6.7: OD absorbance values for 12 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.

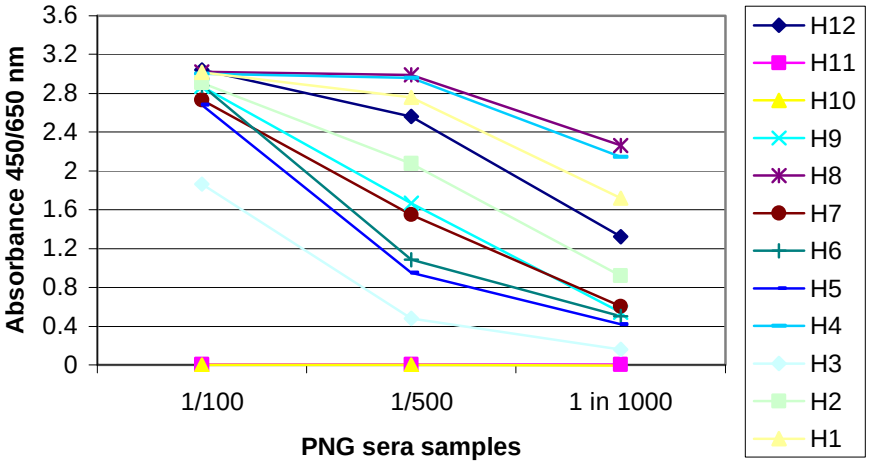

Figure A6.8: OD absorbance values for 12 out of the 90 sera from individuals residing in PNG as measured using the Filariasis CELISA. Reactivity reduced as the dilution increased.