Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 12 April 2018 doi:10.20944/preprints201804.0170.v1

Effects of Lyophilization and Storage Temperature on *Wuchereria bancrofti* Antigen Sensitivity and Stability

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ABSTRACT

Antigen-based rapid diagnostic tests for Lymphatic filariasis do not come with quality control (QC) materials, and research and disease control programmes rely on stored positive plasma samples. This study was undertaken to evaluate the use of freeze-dried *Wuchereria bancrofti* antigen positive plasma samples to serve as QC materials for LF RDTs. 10 well characterized *W. bancrofti* positive samples were lyophilized and stored at 4°C, 28°C and 40°C. The samples were evaluated using the Filaria Test Strips before lyophilization and after one and three (3) months of storage. The sensitivity and stability of the lyophilized samples were evaluated. The results revealed a loss of sensitivity and stability with increasing temperature and duration of storage. The results are further discussed in terms of the use of Dried Blood Spot (DBS) in diagnostics studies on LF requiring quantitative assessments, and the need for thoughtful DBS preparation and storage.

Key words: *Wuchereria bancrofti* antigen, lyophilization, dried blood spots, freeze-drying

INTRODUCTION

Lymphatic filariasis (LF) is found in the tropical and sub-tropical regions of the world, where it is a major public health problem [1]. 74 countries are endemic for the disease, with an estimated 36·5 million global cases of disease in 2016. The availability of tools and strategies for the control of the disease [2-3] led to World Health Assembly resolution (WHA 50.29) calling on member states to work towards the elimination of LF as a public health problem by 2020 [4]. The World Health Organization (WHO) launched the Global Programme to Eliminate Lymphatic Filariasis (GPELF) in 2000, with the principal objective of breaking the cycles of transmission of *Wuchereria bancrofti* and *Brugia spp*. through the application of annual mass drug administration (MDAs) using Ivermectin + Albendazole, DEC + Albendazole or Ivermectin + DEC + Albendazole [5] to entire at-risk populations for a period of 5-7 years. Thus, more than 6·7 billion cumulative treatments were provided to disease endemic communities by 2015 [6]. Impact evaluation assessments are based on the prevalence of infection using *W. bancrofti* antigen rapid diagnostic tests (RDTs) or microscopy for microfilaria presence [7].

As part of the GPELF, millions of RDTs as either the BinaxNOW ICT cards or the Alere FTS kits [8] have been and are currently being used in LF endemic countries without suitable methods for QC. RDT performance can be compromised when test kits are stored for long periods at high temperatures and humidity typical of most endemic countries [9]. Further, these test kits do not come with known positive control materials for their validation, and research and LF control programmes rely on stored samples as QC materials. These samples are stored either in the form of plasma or serum in fridges and freezers, or dried blood spots (DBS) on silica gel and/or in fridge/freezers. This study sought to test the use of lyophilized (freeze-dried) *W. bancrofti* antigen positive plasma, adapted from a similar method used for

 doi:10.20944/preprints201804.0170.v1

HIV rapid tests [10] and malaria [11], as quality control tests for FTS kits. Lyophilization was used because it provides a better temperature and humidity-control during the drying process, compared to DBS [12-13].

Methods

Samples

The Noguchi Memorial Institute for Medical Research (NMIMR) is the premier biomedical research institution in Ghana, and undertakes research on diseases of public health importance, in collaboration with the Ministry of Health and the Ghana Health Service. The samples used for this study are blood samples collected from LF positive individuals, as part of ongoing research activities [14]. Briefly, study participants were tested during the day for LF antigen using the FTS. Individuals positive for antigen were followed for night blood collection (between the hours of 21:00 to 01:00), for the identification of microfilaria. From each participant, 2 ml of night blood was collected (in EDTA coated tubes). 1ml of blood was analyzed for microfilaria using the nucleopore filtration method. The remaining blood was centrifuged, the plasma separated from the pellets, and frozen at -80°C.

Dried W. bancrofti infected blood preparation

For baseline reactivity (six months after storage), 10 frozen plasma samples with known parasite counts (Table 1) were thawed at room temperature and tested on the FTS following the manufacturer's instructions. From the remaining plasma samples, six (6) aliquots (75µl each) per sample were lyophilized using a freeze drier (Lyotrap, Ultra Freeze Dryer LF/LYO/04/1, LTE Scientific). Two aliquots from each of the samples were stored in a refrigerator set at 4°C, at room temperature (28°C +/- 3°C) and in a dry incubator set at 40°C, respectively. These were tested after a period of one and three months. All environmental temperatures are monitored daily.

Dried blood rehydration and FTS testing

On the day of testing (1 and 3 months after storage), the lyophilized samples were rehydrated with 1x PBS solution (pH 7.3). 75µl of PBS was added to the sample tube, and incubated at room temperature for 1 hour. The mixture was gently mixed using a pipette and transferred onto the FTS for reactivity testing.

Reading and scoring of test results

For all tests undertaken (i.e. baseline, 1 and 3 months) the results were scored as negative (test band absent = 0) or positive (test band present). Even though the FTS is a qualitative tool, the intensity of the test line was assessed visually to provide a semi-quantitative score as previously described [15]. Thus, positive results were scored on a scale of 1 to 3; 1 (+) being a test band being lighter in intensity than the control band, 2 (++) being a test band having a similar intensity as the control band, and 3 (+++) being a test band with a brighter intensity than the control band. The control band has to be positive for any test to be valid. A false negative test was defined as *W. bancrofti* infected blood identified as positive by a test at baseline (before lyophilization) and appearing as a negative test after lyophilization. All readings were undertaken and scored by two individuals.

Data analysis

The baseline test results before lyophilization were used as the reference standard, in estimating the sensitivity of using the lyophilized samples. The sensitivity was estimated by combining the results for each temperature storage condition and expressed as:

$$\text{Sensitivity} = \frac{\text{Total samples positive under each storage condition}}{\text{Total samples positive at baseline}}$$

From the semi-quantitative scoring, the mean intensity scores for the baseline, storage temperature and period of storage were computed and test of significance between results were undertaken using the Wilcoxon matched pairs rank test at 95% confidence interval. Graphs and statistical analyses were undertaken using GraphPad Prism version 7.0 (GraphPad Software, Inc., La Jolla, CA).

Ethical considerations

The study received approval from the ethics committee of the Ghana Health Service (GHS-ERC: 04112/2016). It was also reviewed by the NMIMR IRB (CPN 062/16-17) with Federal Wide Assurance Registration (FWA 00001824).

RESULTS

Sensitivity of W. bancrofti lyophilized samples

In this study, 10 samples positive for microfilariae antigen and with known parasite counts were used. Table 1 presents the samples used, their parasite counts, positivity and band intensity level obtained after testing them with the FTS. One sample lost total reactivity after lyophilization, while five others were false negative at 28°C and 40°C at 3 months storage. From the results, 90% sensitivity was observed when the lyophilized samples were tested after one month. After 3 months of storage, the sensitivity of

the lyophilized sample stored at 4°C remained unchanged, while that of the samples stored at 28°C and 40°C decreased to 50% and 60% respectively.

Table 1: Results of FTS testing at baseline, 1 month and 3 months storage at different temperatures

			Results at 1 month			Results at 3 months		
Samples	Microfilaria/ml of blood	Baseline Testing	4°C	28°C	40°C	4°C	28°C	40°C
A	1	+++	+++	+	+++	+++	+	++
B	1	+++	+++	++	++	+	-	+
C	6	+	+	+	+	+	+	+
D	43	++	+	+	+	+	+	-
E	7	+++	++	++	++	++	-	+
F	1	+++	++	++	+	++	+	+
G	6	+++	++	++	++	+	-	-
H	1	++	-	-	-	-	-	-
I	57	+++	+++	+++	++	++	+	+
J	2	++	+++	+++	+++	+	-	-
Number positive		**10**	**9**	**9**	**9**	**9**	**5**	**6**
Sensitivity		**100%**	**90%**	**90%**	**90%**	**90%**	**50%**	**60%**
Mean intensity score		**2.5**	**2.0**	**1.7**	**1.7**	**1.4**	**0.5**	**0.7**
Standard Deviation		**0.707**	**1.054**	**0.949**	**0.949**	**0.843**	**0.527**	**0.675**
Standard Error of mean		**0.224**	**0.333**	**0.300**	**0.300**	**0.267**	**0.167**	**0.213**

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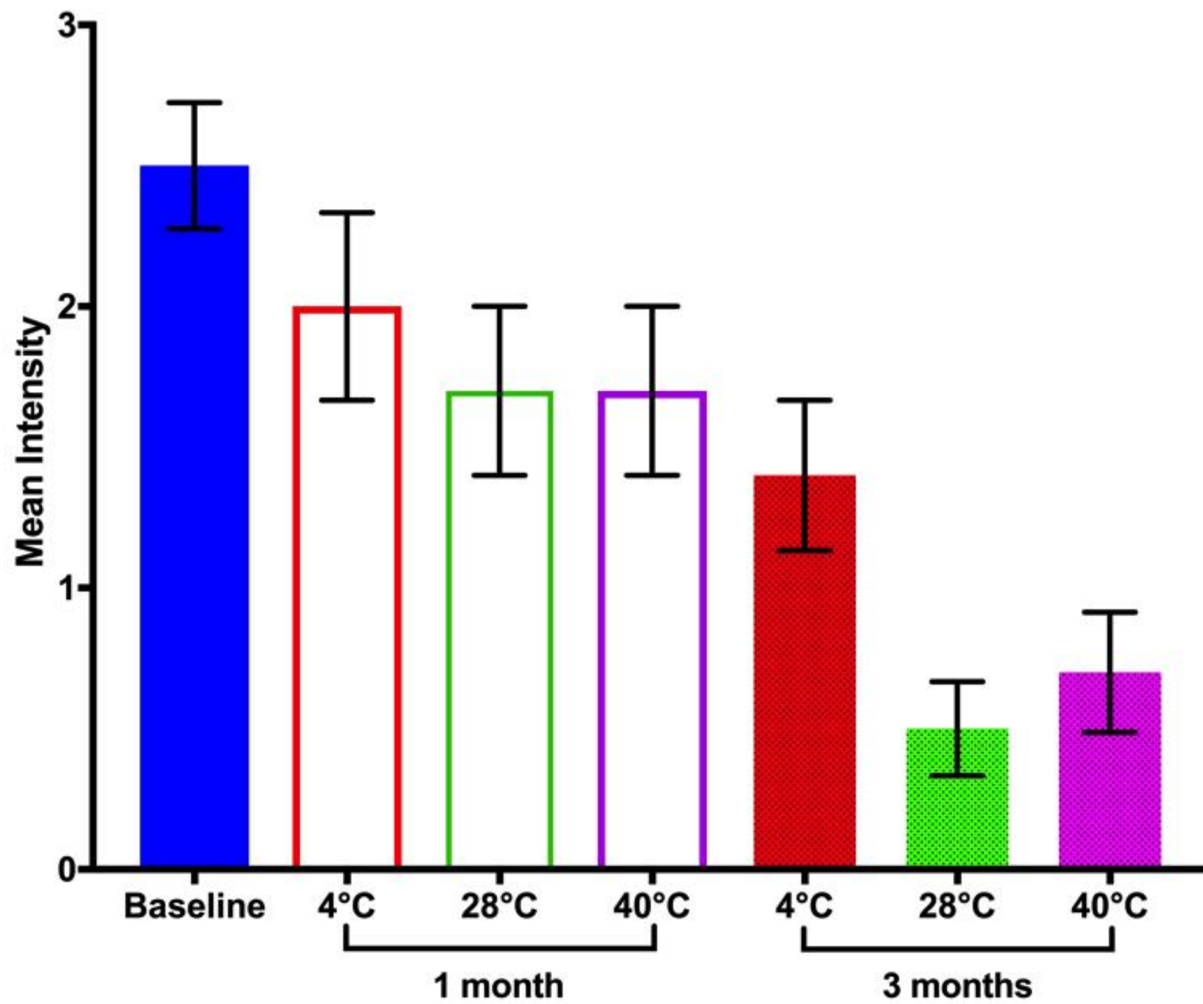

Figure 1: Change in antigen stability with temperature and storage time. Error bars represent the Standard Errors of the Mean.

Assessing W. bancrofti antigen stability

The results showed a decrease in antigen stability with increasing temperature and storage duration (Figure 1). Compared to the baseline, the results indicate a slight but insignificant loss ($p = 0.188$) in

stability in the lyophilized samples stored at 4°C for one month. However, storage at 4°C for three months resulted in further significant loss in stability (p = 0.008) compared to the baseline, and no differences compared to the one month storage (p = 0.125).

Samples stored at 28°C for a month showed an insignificant loss (p = 0.055) in stability compared to the baseline. Storage at three months resulted in a much significant loss compared to the baseline (p = 0.004) and the one-month storage (p = 0.031).

Storing samples at 40°C revealed an insignificant loss in stability (p = 0.055) at one month, compared to the baseline results. However, storage at three months led to a significant loss in stability compared to the baseline (p = 0.004) and the one-month storage (p = 0.016).

DISCUSSION

The results from this study indicate that higher temperatures affect the stability of the *W. bancrofti* antigen. These results also show that long-term storage at higher temperatures result in further loss in antigen stability, with implications for quantitative experiments relying on the use of dried *W. bancrofti* antigen. Studies evaluating the relative performance of plasma and DBS from *W. bancrofti* positive blood samples showed lower positivity and sensitivity for DBS compared to the plasma samples [16], even though both plasma and DBS samples were stored at the same conditions of -20°C (short term) and -80°C (long term). The only difference in temperature conditions was during the drying stage of the DBS and storage of DBS at 4°C or in hand luggage during transportation. Their studies also showed a higher mean antigen concentration in plasma compared to DBS samples. Other studies also showed lower positivity and correlations for DBS compared to serum [17-18], though the storage conditions of the DBS in

these studies was not mentioned. Of course, it is possible the results from these studies could be explained by the fact that less serum (and therefore antigen) is available in the DBS. However, the effects of temperature on further reducing the stability of the antigen in the DBS, as a result of denaturation, must not be ignored.

Our study therefore provides evidence of the effects of drying and temperature on *W. bancrofti* antigen stability. Other studies have shown the effect of temperature on lysosomal enzyme activity during the preparation and storage of DBS [19]. In HIV RDT testing for example, DBS stored at 37°C and 45°C were shown to have good stability until 8 weeks, but when stored at 50°C it showed good stability until week 4. The study concluded that in areas with high temperatures DBS can be stored at room temperature and tested within 4 weeks [20]. In many cases DBS are further stored with dessicants, leading to a further 5% moisture loss during storage [21].

Proteins offer various opportunities in disease treatment and diagnostics. However, in order to maintain their properties, proteins need to be stabilized against physical and chemical degradation [22]. Lyophilization or freeze-drying improves protein-storage stability, ease of shipping and transportation by removing water [23]. While it has the advantages of; being a low-temperature process with less thermo-denaturation risk, controlling moisture level and enabling better stability of proteins, it also has the disadvantage of inducing conformational instability as a result of the freezing and drying process [12-13]. Important characteristics of lyophilized products include long-term stability, short reconstitution time and maintenance of the characteristics of the original products. However for operational purposes, lyophilization is not a process that can easily be carried out during field sample collection, unlike the DBS. It is requires the need and materials for venous blood collection – thus is more intrusive (compared to DBS from finger-prick blood), storage at cold temperatures, a lyophilizer, and as such is

more time consuming and expensive. Nonetheless for quality control purposes, we consider it superior to storage through Dried Blood Spots (DBS), during which, it is challenging to control the moisture content coupled with varying environmental (drying) temperatures – under which LF studies are undertaken – which may result in denaturation and loss of protein/antigen activity. In this study, temperatures of 28°C (+/-3°C) and 40°C were used as these represent the range of temperatures in LF endemic regions in Ghana and possible temperatures under which DBS may be stored in the absence of a fridge or freezer.

Currently, FTS kits are not supplied with positive control materials. Until *W. bancrofti* recombinant positive control antigens become commercially available, research and national control programmes rely on stored samples (where available) as quality control materials. Given the progress made through GPELF and the low/limited numbers of positive individuals being detected, sample storage methods should carefully be considered if these are to serve as positive control materials for future studies and programme evaluation activities, most especially for studies relying on quantitative assessment methods and the determination of positive cut-off thresholds. While the use of dried parasitized blood has been evaluated as QC materials for HIV [10] and malaria [11] tests, and may be applicable to LF, further developments of the method aimed at stabilizing the antigen (e.g. through the addition of dissacharide sugars) [24], followed by field testing may enhance the utility of lyophilized *W. bancrofti* antigen in the future.

The main challenge to this study was the mall number of samples evaluated. This is due to challenges in obtaining large numbers of well-characterized samples at the current stage of the GPELF in Ghana. While other samples positive with *W. bancrofti* day blood antigen are available, sample size was limited to 10 in order to avoid using all samples at the expense for future positive control materials. The sample

volumes available also prevented the preparation of DBS for comparison. Another challenge to the FTS reading may also be the subjectivity of the readings by the testing personnel, especially for very faint test bands. However, having two personnel assess the results helped resolve the challenges.

In conclusion, the results from this study would suggest that dried *W. bancrofti* antigen (either lyophilized or on DBS) stored for long periods above 4°C should carefully be used in quantitative experiments, given the loss in antigen sensitivity over time. While the DBS is a simpler and cheaper method than collecting samples in EDTA tubes, care must be taken in the storage of these samples in order to obtain the best possible results from their use.

Acknowledgement: We are grateful to Prof. Regina Appiah-Opong for freeze-drying the samples for us. This project was undertaken as part of the EDCTP2 programme supported by the European Union, through grant number 98595 to DKdS. The funding source has no role in the design of the study, in the collection, analysis and interpretation of the data, or in the publication of the study results.

Author Contributions: D.K.dS. and D.A.B. conceived the study. E.Y.A., E.D. and D.K.dS designed the experiments. E.Y.A. and E.D. performed the experiments. E.Y.A. and D.K.dS. analyzed the data. E.Y.A., E.D., D.A.B. and D.K.dS wrote the paper. All authors reviewed and approved the final draft.

Conflict of Interest: The authors declare no conflict of interest.

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