EVALUATION OF THE ACCURACY OF THE CD4+ T-CELL COUNTS USING PIMA™ CD4 AS COMPARED TO BD FACSCALIBER FLOW CYTOMETRY

KAFUFU FRED BOSCO (MLS 2008/HD17/13909U) (203003412)

A dissertation submitted to School of Graduate Studies in partial fulfillment of the requirements for the award of Master of Science Degree in Biomedical Laboratory Sciences and Management of Makerere University

May 2010

DECLARATION

This research dissertation has been submitted for examination with the approval of the supervisors:

Dr. Jesca Lukanga Nakavuma (PhD)

Microbiologist and Head

Department of Veterinary Parasitology and Microbiology,

Faculty of Veterinary Medicine

Makerere University

P.O. Box 7062 Kampala Uganda

Signature Date.....

Dr. Emmanuel Othieno (MBChB, MSc MED Pathology)

Senior Consultant Clinical Pathologist

Dept of Pathology, College of Health Sciences

Makerere University

P.O. Box 7062 Kampala Uganda

Signature..... Date.....

DEDICATION

I dedicate this thesis to my father Ssalongo Ssajjabi Dawson, who taught me that the best kind of knowledge to have, is that which is learned for its own sake. To my mother Agnes Nakiranda, who taught me that even the largest task can be accomplished if it is done one step at a time. It is also dedicated to my son Alvin Blessing Muwanguzi, born pre-term and had a brain surgery at 4 days old. I almost withdrew from this course at the beginning of the first semester of 2nd year, but after a successful operation he gave me hope to continue. God should be glorified and be pleased with all of them.

ACKNOWLEDGEMENT

I would like to thank all people who have helped and inspired me during my Master's study. I especially want to offer my sincere gratitude to my supervisors Dr. Jesca Lukanga Nakavuma, who has supported me throughout my thesis with her patience and knowledge while allowing me the room to work in my own way. I attribute the level of my Masters degree to her encouragement and effort and without her, this thesis, would not have been completed. I was delighted to interact with Dr. Emmanuel Othieno by attending his classes and having him as my supervisor. His guidance, encouragement, supervision and support from preliminary to the concluding level enabled me to develop an understanding of the subject. In my daily work I have been blessed with several lifelong friends especially Dr. Godfrey Kagezi, who lent me his laptop to use till the end of my course. I am indebted to him more than he knows. I am grateful to Dr. Mugasa Claire, for her guidance during my research and seminar presentations. Her perpetual energy and enthusiasm in research had motivated all her students, including me. Besides, she was always eligible and willing to help students with their research. My deepest gratitude goes to my family for their unflagging love and support throughout my life, this dissertation is simply impossible without them. Words fail me to express my appreciation to my dear wife Teopister Muwanguzi whose dedication, love and persistent confidence in me, has taken the load off my shoulder. I owe her for being unselfishly let her intelligence, passions and ambitions collide with mine. I acknowledge the infectious Diseases Institute as my research was supported in part by them. Finally, I offer my regards and blessing to all our friends (HIV positive patients are fondly called friends at IDI)) attending the adult HIV/AIDS clinics at IDI for accepting to take part in this study.

TABLE OF CONTENTS

DECLARA	TION	i
DEDICATI	ON	ii
ACKNOWI	LEDGEMENT	.iii
TABLE OF	CONTENTS	. iv
LIST OF FI	GURES	vii
LIST OF TA	ABLES	. ix
LIST OF AG	CRYNONYS AND ABBREVIATIONS	X
ABSTRACT	Г	. xi
CHAPTER	ONE: INTRODUCTION	1
1.1	Background	1
1.2.	Problem statement	3
1.3.	Justification of the study	4
1.4.	General objective	5
1.5.	Specific objectives	5
1.6.	Research Questions	6
CHAPTER	TWO: LITERATURE REVIEW	7
2.1.	Human Immunodeficiency Virus (HIV)	7
2.2.	CD4+ T-lymphocytes	9
2.4.	CD4 T lymphocytes counts in pediatric population	13
2.5	CD4 T-lymphocytes counts in pregnant women	15
2.6.	CD4 count and CD4 percentage in HIV-infected people	16
2.7.	Factors that influence a CD4 cell count	18
2.8.	CD4 count in resource limited settings	20
2.9.	CD4 counting methods for HIV monitoring	21

2.9.	.1.	Flow cytometers
2.9.	.2.	Pima CD4
2.10.	Eval	luating test assay precision25
2.11.	Ce	ll gating
CHAPTER	R THE	REE: MATERIALS AND METHODS 30
3.1.0.	Stud	y design 30
3.1.	.1.	Study site
3.1.	.2.	Sample size
3.1.	.3.	Study population
3.1.	.4.	Inclusion criteria of study participants
3.1.	.5.	Exclusion criteria of study participants
3.2.	.0	Study materials
3.3.	.0	Laboratory analysis (sample collection and CD4 T-cell analysis) 33
3.3.	.0.	Quality Control (QC)
3.4.	.0.	Accuracy of CD4 counting on the Pima CD4
3.5.	.0	Assessment of precision of CD4 counting
3.6.	.0	Types of errors and their frequencies
3.7.	.0.	Ethical approval
3.8.	.0.	Statistical analysis
CHAPTER	R FOU	JR: RESULTS
4.1.	Ch	aracteristics of the study population
4.2.	CE	04 counts for the various age groups of the patients by gender using
	Pir	na CD4 for venous blood
4.3.	CE	04 counts in pregnant and non-pregnant female patients

4.4.1	Comparison of results using BD FACSCaliber and Pima CD4 on venous
	whole and capillary blood
4.4.1.	Linear regression techniques for BD FASCaliber and Pima CD4 46
4.4.2.	Bland-Altman techniques for BD FASCaliber and Pima CD4
4.3	Precision of T-cell counts obtained by using the Pima CD4 test on whole
	blood samples
4.5	Types and frequency of errors incurred by operators when using Pima
	CD4
CHAPTER	FIVE: DISCUSSION
CHAPTER	SIX: CONCLUSIONS AND RECOMMENDATIONS 67
6.1.	Conclusions
6.2.	Recommendations
REFERENC	CES
Appendix I:	Gating of anticipated cell population distribution:
Appendix II	: Study Schema
Appendix II	I: RESEARCH SUBJECT INFORMATION SHEET
Appendix I	V: Facscalibur daily operations SOP92
Appendix V	2: QUICK REFERENCE GUIDE
Appendix V	I: QUICK REFERENCE GUIDE SAMPLE COLLECTOR
Appendix V	II: Ethical Approval 102

LIST OF FIGURES

Figure 1	Mean CD4 counts distribution in age groups for Pima CD4 using
	venous blood 40
Figure 2	CD4 counts in pregnant and non-pregnant female patients by the
	different testing instruments and blood source
Figure 3	Comparison of results using BD FACSCaliber using and Pima CD4 on
	venous and capillary blood for the various CD4 count ranges
Figure 4	Agreement of mean CD4 counts for BD FACSCaliber using venous
	whole blood and Pima CD4 using venous whole and capillary blood
	for the various CD4 count ranges
Figure 5	Agreement of mean CD4 counts for BD FACSCaliber using venous
	whole blood and Pima CD4 using venous and capillary whole blood
	for the various age groups
Figure 6	Correlation analysis of BD FACSCaliber and Pima CD4 using venous
	blood for CD4 cell counts (cells/µl.)
Figure 7	Correlation analysis of capillary blood using Pima CD4 with whole
	blood obtained by BD FACSCaliber
Figure 8	Correlation analysis of Pima CD4 using capillary and venous whole
	blood
Figure 9	Bland-Altman plot comparing the mean and the difference between
	standard BD FACSCaliber and Pima CD4 cell counts using venous
	whole blood
Figure 10	Bland-Altman plot comparing the mean and the difference of capillary
	blood using Pima CD4 with whole blood obtained by BD FACSCaliber

Figure 11	Bland-Altman plot comparing the mean and difference of Pima	CD4
	using capillary and venous whole blood	52

Figure 12	Within-run (Plot A) and Between-run (Plot B) precision for Pima	CD4
	results for sample 1 and 2 and Normal and Low CD4 count sam	mples
	respectively	54

Figure 13	Types and frequency (%) of errors committed by operator when	using
	Pima CD4 venous and capillary blood	55

LIST OF TABLES

Table 1:	Distribution of the study patients by age and gender	39
Table 2	CD4 counts in pregnant and non-pregnant female patients by t	the
	different testing instruments and blood source	42
Table 3	Agreement of mean CD4 counts between Pima CD4 and H	3D
	FACSCaliber for the CD4 ranges	45
Table 4	Summary for precision of Pima CD4	53

LIST OF ACRYNONYS AND ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
ART	Anti-Retroviral Therapy
BD	Becton-Dickinson
CD	Cluster of designation
CDC	Centers for Disease Control
CI	Confidence Interval
CL	Confidence Limit
CLSI	Clinical Laboratory Standards Institute
FDA	Food and Drug Administration of the US
HIV	Human Immunodeficiency Virus
IRB	Institutional Review Board
K ₂ EDTA	Ethyele-diamine-tetra-acetic acid, di-potassium Salt
МТСТ	Mother to Child Transmission
Ml	Milliliters
MMWR	Morbidity and Mortality Weekly Report
SDI	Standard Deviation Index
SOP	Standard Operating Procedure
UVRI	Uganda Virus Research Institute
WHO	World Health Organisation
μL	Microliters

ABSTRACT

Introduction

Pima CD4 (Inverness Medical group 2009), a new method for enumerating CD4+ T-cells is affordable, technically simple, and economical in that it can use either electricity or battery. It is fully automated and thus, useful in remote settings. However, limited information on its performance exists in current literature. Thus, this study aimed at assessing the accuracy of Pima CD4 to BD FASCaliber flow cytometer (Becton Dickinson, Franklin Lakes and NJ) and precision of CD4+ T-cell counting using freshly collected capillary and venous whole blood samples, and establishing the types and frequency of errors incurred when using Pima CD4. This study reported the performance of the relatively new Pima CD4 laboratory test as compared to the more established FASCaliber flow cytometer in determination of the CD4 counts in HIV-infected individuals. The study addressed an important effort in up-scaling rapid, efficient and reliable means of management of HIV in resource limited setting such as in rural Uganda.

Materials and methods

Compared results of absolute CD4 counts obtained on replicate samples from 206 HIV-infected individuals (adult men and women) by Pima CD4 with those generated by BD FASCaliber flow cytometry at the Infectious Diseases Institute in Kampala, Uganda.

Results

Using venous whole blood, the mean CD4 counts were higher for BD FACSCaliber $(422\pm220 \text{ cells/}\mu\text{L})$ but not significantly different (P=0.1289) from that for Pima CD4 $(391\pm201 \text{ cells/}\mu\text{L})$. Results from the two machines were highly positively correlated (r = 0.96). The mean CD4 counts for BD FASCaliber using venous whole blood

(422 \pm 220 cells/µL) were significantly higher (P=0.0116 at p=0.05 level) than for Pima CD4 using capillary blood (371 \pm 185 cells//µL). Further, the mean CD4 counts enumerated by Pima CD4 for venous whole blood (391 \pm 201 cells//µL) were higher than that in capillary blood (371 \pm 185 cells//µL) but not significantly different (P=0.3142). Further, within run precision demonstrated that the inherent imprecision of the Pima CD4 instrument is within the manufacturer's claims and clinically acceptable limits. Also, between run precision demonstrated that the overall inherent imprecision of Pima CD4 instrument and due to other external variants is within manufacturer's claims and clinically acceptable limits. Using venous whole blood the operator committed 24.3% errors with channel filling and reagent quality control being the most prominent. Likewise, when using capillary blood, the operator committed 13.6% errors with image and reagent quality control being the most prominent.

Conclusions/recommendations

Despite the few short comings, Pima CD4 maybe currently one of the suitable instruments for health centers and remote areas with limited access to CD4 testing centers. However, there is still need for proper and thorough training of the operators. Lastly, this study was absolutely done in laboratory environment. Thus, there is a need to evaluate the Pima CD4 in field conditions particularly in remote areas.

CHAPTER ONE: INTRODUCTION

1.1 Background

The enumeration of CD4+ T-Lymphocytes in the peripheral blood is an essential tool for the laboratory monitoring of HIV infected patients in terms of progression of disease and for assessment of outcome of anti-retroviral treatment (ART). The ART programme initiated at this scale would require extensive back-up for counseling, laboratory investigations to support initiation and monitoring of ART and clinical management of adverse reactions. Important decisions like when to start anti-retroviral therapy or prophylaxis for opportunistic infections are dependent on the CD4+ T cell count estimation. However, in the absence of facilities for viral load assays, CD4+ T cell count estimation is used for monitoring anti-retroviral therapy (Kovacs and Masur, 2003). There is therefore need for providing reliable CD4+ T cell counts for successful HIV patient care and treatment programme. Although viral load testing provides the most accurate assessment of response to ART, its routine use is prohibitively expensive in resource-limited settings. For this reason, WHO supports CD4 cell count measurement to monitor response to ART (WHO, 2007). The 2006 WHO guidelines define CD4 cell failure as persistent CD4 levels below 100 cell/mm³ or fall of CD4 count to pre-therapy baseline or 50% fall from the on-treatment peak value without concomitant infection to cause transient CD4 cell decrease.

The primary laboratory-based method of HIV disease staging is measurement of CD4 lymphocytes, which is the most reliable indicator of the risk for opportunistic infections and is used to initiate ART and opportunistic infection (OI) prophylaxis (WHO, 2007). Flow cytometry, though a very commonly used method in determining CD4+ T-cell measurements since the beginning of HIV epidemic, is

expensive in terms of initial running costs and need for skilled manpower hence limiting its use (Brando *et al.*, 2000; Crowe *et al.*, 2003). In addition, flow cytometric tests should be conducted in sophisticated referral laboratories and by highly trained medical technologists; hence is not easily employed in the resource poor settings. Due to the ever-increasing demand for CD4+ T-cell testing, particularly in resourcelimited countries, there is need to decentralize CD4+ test at the point-of-care to greatly improve access to CD4 tests. In addition, these tests should be performed in the simplest circumstances in the remote parts of countries with a wide range of climates by trained healthcare workers with limited experience in performing laboratory tests (Nicholson *et al.*, 1994; Pattanapanyasat and Thakar, 2005).

With FACSCaliber flow cytometry being very expensive, alternative and more affordable CD4 counting methods have been developed especially for use in resource-poor settings. These methods include improved cytometric approaches, microbead capture of CD4 cells followed by manual cell counting; and prototype microchip counting methods and recently the PIMA CD4 counting equipment, which employs fluorescein-tagged specific antibodies to T-helper cell markers (Landay, *et al.*, 1993; Sherman, *et al.*, 1999; Kannangai, *et al.*, 2001; Janossy G, *et al.*, 2002b; Rodriguez, *et al.*, 2005; Bentwich, 2005; Inverness Medical Group, 2009). Most of these assays are cheaper than the regular flow cytometry but suffer from decreased accuracy, precision and have a low throughput.

Pima CD4 system is much simpler to use, is light and easily carried by the operator (mobile) into a clinic. Unlike other CD4 test methods, it does not require liquid biohazardous waste disposal. Pima CD4 is more affordable, technically simple, and economical in that it can use

either electricity or battery, and is fully automated (Inverness Medical group, 2009). The performance of Pima CD4 was evaluated in South Africa in a laboratory setting (Glencross *et al.*, 2010) and the results compared favorably with those of the reference method although they recommended assessing the tool using finger stick blood samples and also in the field sites using operators other than the laboratory technologists. A similar study on Pima CD4 machine was done in Germany and Uganda (UVRI) by Inverness Medical Group (2009) to establish the accuracy, clinical agreement and lot-lot reproducibility. Like the South African study, the test samples were drawn from satellite clinics and transported to the testing sites; yet the factors that influence the variability of CD4 counts may include storage and transport temperature, time and age of samples (Peter *et al.*, 2008). Following the recommendations and gaps identified from the above studies, the current study was conducted in a designated hospital clinic to address accuracy, precision of CD4 counts using capillary finger stick and fresh venous whole blood on the Pima CD4.

1.2. Problem statement

There are increasing numbers of HIV infected individuals in Uganda; and evaluation for when to initiate therapy is mainly based on clinical assessment, which is often too late to enable them benefit from the treatment. This is due to the fact that by this time, the disease is fully blown. CD4 cell count which is used as a predicator of disease progression, is the most reliable indicator of the risk for opportunistic infections (OI), and therefore, used for decision to initiate ART in both adults and children (WHO, 2006). The reference standard method for measuring CD4 lymphocytes is flow cytometry; and the available testing methods, such as FACSCAliber System (Becton Dickinson), are quite not applicable in rural settings due to the cost (\$30,000 to \$150,000), technical and operational complexity, and the need for reliable electricity, and the high cost of reagents. All these factors have made these instruments impractical and/or difficult to sustain in resource-scarce settings. Hence, there is limited access to facilities with CD4+ cell testing capacities, which is a hindrance to many HIV/AIDS patients from receiving appropriate care. Therefore, in resource-limited settings, there is need for adoption of cheaper and technically simpler alternatives to enumerate CD4+ cells, since testing is a continuous process that should be repeated every 3-6 months for efficient administration of ART (Rodriguez *et al.*, 2005)

1.3. Justification of the study

A dramatic increase in demand for CD4 counts especially in resource-limited settings (WHO, 2003) prompted an investigation for a more affordable and technically simple alternative to access CD4 counts. It is believed that these tests might be performed within either referral laboratories (for instance using FACSCAliber flow cytometry) or the simplest circumstances in the remote parts of countries with a wide range of climates (for example by using Pima CD4). An alternative to the expensive FACSCAlibur flow cytometry that could potentially offer solutions for CD4 testing in whole blood is the Pima CD4. Pima CD4 is more affordable, technically simpler and economical in that it can use either electricity or battery, is fully automated and thus, useful in remote areas. However, there is limited information regarding the performance of Pima CD4 in enumerating CD4+ T-cell in whole blood of HIV-positive individuals. Previous studies in the Ugandan setting employed test samples collected from satellite clinics and transported to the testing sites; hence the factors that influence the variability of CD4 counts such

as storage and transport temperatures; time and age of samples; may have influenced the outcomes.

Thus, this study aimed at assessing the accuracy and precision of CD4+ T-cell counting using freshly collected capillary and venous whole blood samples, and establishing the types and frequency of errors incurred when using Pima CD4. The availability of a CD4+ T-cell test on capillary fingerstick blood that can be performed in the physician's office would increase the ease of CD4+ T-cell testing; reduce patient discomfort and turn around time. The findings from this study will go a long way in addressing the needs of manufacturers, policy makers and the population in rural settings at primary health centres by providing decentralized CD4+ test at the point-of-care.

1.4. General objective

The primary aim of the study was the evaluation of the accuracy of the CD4+ T-cell counts in whole blood samples using Pima[™] CD4 as compared to the cell counts obtained on whole blood samples from the same individuals using BD FACSCalibur flow cytometer as a reference method

1.5. Specific objectives

- To establish the agreement between CD4+ T-cell counts obtained by Pima CD4 and BD FACSCaliber using venous whole blood.
- 2. To establish the difference between CD4+ T-cell counts obtained by Pima CD4 using capillary blood and BD FACSCaliber using venous whole blood.
- 3. To demonstrate the equivalence of CD4+ T-cell counts in capillary blood with that in venous blood obtained by Pima CD4 equipment

- 4. To assess the precision of T-cell counts obtained by using the Pima CD4 test on whole blood samples.
- To determine the types and frequency of errors incurred by trained operators when using a Pima CD4.

1.6. Research Questions

- Is there an agreement between CD4+ T-cell counts obtained by Pima CD4 and BD FACSCaliber using venous whole blood?
- Is there a significant difference in CD4+ T-cell counts in capillary blood using Pima CD4 with that of venous whole blood obtained by BD FACSCaliber?
- 3. Is there any equivalence of CD4 T-cell counts in capillary blood with that in venous blood using the Pima CD4 equipment?
- 4. Is the precision of the T-cell counts obtained by using the Pima CD4 test on whole blood samples within manufacturer's claims and clinically acceptable limits?
- 5. What are the common types of errors and their frequency when using a Pima CD4?

CHAPTER TWO: LITERATURE REVIEW

2.1. Human Immunodeficiency Virus (HIV)

Since the first case of HIV was reported way back in early1981 (CDC, 1981), HIV infection has reached pandemic proportions in many countries worldwide. Statistics indicate a growing number of infections. For instance by the end of 2007, about 33.2 million people were believed to be living with HIV worldwide, out of these, there were 2.5 million new infections and 2.1 million deaths due to HIV related illnesses thus full blown AIDS. As the world population grows, more people continue to get infected, but with the introduction of antiretroviral drugs, such people will be facilitated to live longer (UNAIDS/WHO 2003, 2007). The catastrophe has greatly impacted on both human life and the economic and health basics in countries with high HIV prevalence (Quinn, 2003). The HIV prevalence in Uganda is estimated to be 5.4% amongst adults. The number of people living with HIV is higher in urban areas (10.1%) than the 5.7% found in rural areas. It is also important to note that the prevalence is higher among women (7.5%) compared to men (5.0%) (UNAIDs, 2008)

Antiretroviral therapy (ART) availability has increased considerably in sub-Saharan Africa, thus increasing the importance of laboratory methods, which influence decisions for initiating treatment and monitoring response to therapy. This is being initiated by both Government and non-governmental organizations who are working towards increasing availability and reduction in the costs of laboratory tests. The various organizations include, the Clinton Foundation HIV/AIDS Initiative which in 2004 entered an agreement with companies, including Beckman Coulter (CD4 lymphocyte count equipment manufacturer) and Roche Diagnostics (manufacturer of reagents for HIV viral load determination), in order to lower the laboratory test prices

than the current market rates in resource-limited settings (Deghaye *et al.*, 2006). However, in spite of reducing the cost of diagnostics, there is more to this issue than meets the eye because the process stretches further to personnel, medication, and laboratory tests (Deghaye *et al.*, 2006). In South Africa for example, laboratory costs alone comprise 50% of costs before ART and during the first month of treatment (Deghaye *et al.*, 2006). Hence there is a need for lower cost assays that do not compromise the quality of results. In this way there would be a major impact in costs of HIV/AIDS patient care.

HIV disease staging allows healthcare providers to ascertain which patients might benefit most from ART and opportunistic infection (OI) prophylaxis. The World Health Organization has published guidelines for initiation of ART based on clinical signs and CD4 lymphocyte count (WHO, 2006). Since funds for laboratory tests may be unavailable in resource-limited settings, HIV disease staging is often limited to a clinical assessment of the patient for signs and symptoms of OI. However, clinical expression of HIV-1 infection is often a late-stage event that can be fatal. Hence earlier identification of persons who are at risk for morbidity and mortality but without clinical signs or symptoms requires laboratory assessment (Mayanja-Kizza *et al.*, 2005). Routine clinical markers lack the relevant sensitivity and hence cannot replace CD4 cell count and HIV viral load as indicators of successful HIV treatment (Bagchi *et al.*, 2007).

A number of intervention strategies are in place among which include the reduction of viral load by efficient anti-retroviral therapy (ART), and this has been facilitated by governmental and non-governmental organizations who are working towards increasing supply of such ART. Increase in ART must be coupled with access to disease monitoring; hence the enumeration of CD4+ T-Lymphocytes in the peripheral blood serves as an essential tool for monitoring of HIV infected patients (Pattanapanyasat and Thakar, 2005).

2.2. CD4+ T-lymphocytes

These are a type of lymphocytes (white blood cell) that play an important role in the immune system by controlling the body's ability to recognize and fight infections and cancers (Norris and Rosenberg, 2002). These infections can be bacterial, fungal, viral or parasitic in origin. The lymphocytes also regulate the production of antibodies (proteins that fight infections) and cytokines (chemicals that regulate other immune functions) (HIV) (Hogan and Hammer, 2001). The cells are attacked by HIV virus which enters them, or binds to surface and reproduces immediately and the consequence is either cell death or the viral genome is integrated or virus remains dormant till the cells become active again. Much as the body attempts to produce new lymphocytes to replace the ones that are destroyed, the number of CD4 cells eventually declines as HIV disease progresses (Hogan and Hammer, 2001). This makes the CD4 cell count an important test for following up HIV infected patients because it is a laboratory marker of the strength of the immune system. It also helps to determine how advanced the HIV disease is and in the process predict the risk of complications (Peto, 1996).

HIV infect cells displaying the CD4 cell surface protein but also other cells such as Bcells, certain brain and intestinal cells but more commonly they infect macrophages and T-helper (T_H) cells. The infected macrophages and T_H cells produce and release large numbers of HIV particles which in turn infect other cells that display CD₄ proteins. Infection normally occurs first in macrophages, which are the antigen presenting cells (APC) that have a low level of CD_4 proteins on their surfaces. The macrophage CD₄ molecule binds to the glycoprotein gp120 protein of HIV, which then interacts with another macrophage protein, the membrane spanning chemokine receptor (CCR5), which acts as a co-receptor for HIV and together with CD₄ they form the docking site where HIV envelope fuses with the host cell membrane, allowing insertion of the viral nucleoprotein. Individuals who express a variant of CCR5 protein do not bind HIV and do not acquire HIV infection or AIDS. After infecting the macrophage APCs, a different form of gp120 is made, which in turn binds to a different co-receptor, the CXCR4 chemokine receptor, on T cells. HIV then enters and destroys the CD₄ T_H (Th1 and Th2 cells) responsible for cell mediated inflammatory response and B-cell helper respectively. This results in systemic destruction of macrophages and T-cells leading to breakdown of immunity. The infected T-cells process HIV antigens and insert molecules of gp120 from HIV particles into their cell surface and this facilitates aggregation of uninfected cells to the infected ones and fusion land hence producing multinucleate giant cells or syncytia. One HIV-infected T-cell may eventually bind and fuse with up to 50 uninfected T-cells. The end-result of HIV infection is decline in CD₄ cell numbers, thus opportunistic infections become established. CD₄ cells decline is concomitant with loss in cytokine production leading to gradual reduction in uninfected T-cells, eventually all other lymphocyte production is shut down and immune system is effectively destroyed (Crowe et al., 1987; Pugach et al., 2004).

All in all, the CD4+ T-cell count and viral load are the most important marker best used in initiation and monitoring of ART and hence a measure of the effectiveness of treatment in clinical evaluations (Pattanapanyasat and Thakar, 2005). Guidelines have therefore been developed to address the quality control of CD4+ testing in persons with HIV infection (Rachlis and Zarowny, 1998).

2.3. HIV/AIDS clinical staging

The different stages of HIV infection can generally be broken down into four distinct stages: Clinical stage 1 (clinically asymptomatic stage), Clinical stage 2 (mild disease) Clinical stage 3 (moderate disease) and Clinical stage 4 (progression from HIV to AIDS). Guidelines on clinical staging of HIV-associated disease to be used in patients with confirmed HIV infection were designed by World Health Organization (WHO, 2006, 2007)

Clinical stage 1 is clinically asymptomatic stage and, as its name suggests, is free from major symptoms. The patient carries a disease or infection but experiences no symptoms. The level of HIV in the peripheral blood drops to very low levels but people remain infectious. A condition might be asymptomatic if it fails to show the noticeable symptoms with which it is usually associated. Asymptomatic infections are also called subclinical infections. In this stage there may also be swollen glands or persistent generalized lymphadenopathy (PGL). This however, over time culminates in stage two.

Clinical stage 2 of HIV infection is often characterized by multi-system disease and infections can occur in almost all body systems. There is unexplained weight loss (<10% of presumed or measured body weight); and continually suffers from persistent respiratory tract infections (such as sinusitis, bronchitis, otitis media,

pharyngitis) and oral ulcerations. At this stage as the immune system fails, herpes zoster, papular pruritis eruptions, angular cheilitis, seborrhoeic dermatitis and fungal finger nail infections set in. Initially many of the symptoms are mild, but as the immune system deteriorates the symptoms worsen.

Clinical stage 3 (moderate disease) sets in as the immune system continues to be weakened by HIV and at this stage moderate opportunistic infections and cancers that the defence system would normally prevent emerge. Signs and symptoms become more prominent. The symptoms described as mild in stage two gets more severe. Here unexplained malnutrition not adequately responding to standard therapy and unexplained severe weight loss (>10% of presumed or measured body weight) are more severe. Sicknesses such as diarrhea are more prominent and tend to last longer than expected. Other conditions where a presumptive diagnosis can be made on the basis of clinical signs or simple investigations include: unexplained persistent fever (above 37.5°C, intermittent or constant, for longer than one month), persistent oral candidiasis, oral hairy leukoplakia (OHL), and pulmonary tuberculosis. Clinical stage three is also characterized by severe bacterial infections (such as pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia), acute necrotizing ulcerative stomatitis, gingivitis or periodontitis and unexplained anaemia (<8 g/dl), neutropenia (<0.5 \times 109 /litre) or chronic thrombocytopenia (<50 \times 10 9 /litre).

Clinical stage 4 (severe disease) occurs when the immune system becomes more and more damaged; the illnesses that occur become more and more severe leading eventually to an AIDS diagnosis. The features that were recurring in clinical stages 1, 2 and 3 become almost permanent wasting away the victim. This is simply called HIV wasting syndrome coupled with Pneumocystis jirovecii (formerly Pneumocystis carinii) pneumonia (PCP) and recurrent severe bacterial pneumonia. There is also chronic herpes simplex infection (orolabial, genital or anorectal, which last more than one month), oesophageal candidiasis (or candidiasis of the trachea, bronchi or lungs) which break down the respiratory system accelerating extrapulmonary TB (EPTB). Patients with HIV may also develop one or more of the following: Kaposi sarcoma, cytomegalovirus (CMV) infection (retinitis or infection of other organs), toxoplasmosis of the central nervous system (CNS), extrapulmonary cryptococcosis disseminated non-tuberculous mycobacterial infection, including meningitis, multifocal leukoencephalopathy (PML), Penicilliosis, progressive chronic disseminated cryptosporidiosis and isosporiasis, mycosis (extrapulmonary histoplasmosis, coccidiodomycosis); recurrent septicaemia (including those due to non-typhoidal Salmonella), lymphoma (cerebral or B-cell, non-Hodgkin), invasive cervical carcinoma, atypical disseminated leishmaniasis, symptomatic HIV-associated nephropathy or HIV-associated, and cardiomyopathy

2.4. CD4 T lymphocytes counts in pediatric population

In countries with a high seroprevalence of human immunodeficiency virus type 1 (HIV-1), infection contributes significantly to infant mortality. Infants with human immunodeficiency virus type 1 (HIV-1) infection have much higher rates of disease progression and mortality than older children. Infants less than 18 months of age have higher total lymphocyte counts and absolute CD4 T lymphocytes counts than those in the adult population. Hence percentage levels of CD4 are the preferred measurement

in children less than five years old, as it varies less in them than in older children. After five years of age, either CD4 % or absolute CD4 count can be used but the latter is preferred. The total leucocyte count (TLC) is an option that is used only if CD4 measurement is not available in children with HIV/AIDS clinical stage 2 disease. The TLC cannot be used in asymptomatic children and is also not useful for monitoring ART (WHO, 2007).

Whereas early initiation of antiretroviral therapy may be appropriate for infants, continuing treatment for life is problematic, given the limitations of the available drugs, that is, the long-term toxicity of antiretroviral therapy; adherence issues; the risk of resistance to antiretroviral therapy; and limited resources. Major challenges in delivering treatment include the lack of paediatric ARV formulations that can be dosed in small children and limited studies examining safety and efficacy for those that are available. In contrast to adult studies, paediatric studies often enroll smaller numbers of patients, which limits the interpretation of the available data. HIV-positive children continue to be 'therapeutic orphans' and paediatric ARVs that are safe, tolerable, efficacious and simple to use are urgently needed in resource-limited settings (ITPC, 2007). Another challenge is that most serological methods used to diagnose HIV are not reliable for children under 18 months. Confirmation by virological tests is needed but these tests are expensive, need sophisticated laboratory facilities and thus are not readily available (Ekpini and Gilks, 2005; WHO, 2006, 2007; Bowen *et al.*, 2008).

2. 5 CD4 T-lymphocytes counts in pregnant women

In sub-Saharan Africa, 14 million women of childbearing age are living with HIV/AIDS, and the risk of mother-to-child transmission (MTCT) of HIV during pregnancy, delivery or breastfeeding is 15-45% (WHO, 2004a). Antiretroviral drugs play an important part in the interventions aimed at reducing the risk of HIV MTCT. At present, the type and number of antiretroviral drugs needed, together with the foreseeable antiretroviral treatment duration partly depend on the mother's CD4 cell risk count (WHO, 2004a). The of MTCT rises with the increasing immunosuppression of the mother, and whether a pregnant woman meets the criteria for receiving highly active antiretroviral therapy (HAART) largely depends on her immunological status (Leroy et al., 2002; WHO, 2004a).

CD4 counts have been reported to be lower in pregnant than in non-pregnant women in HIV-positive population (Burns *et al.*, 1996; Danisman *et al.*, 2001) as well as in HIV-negative ones (Bisalinkumi *et al.*, 1992; Dayama *et al.*, 2003; Aina *et al.*, 2005; Chama *et al.*, 2009). Both laboratory and clinical studies have shown that Pregnancy is associated with altered immunity which could enhance the immunosuppression associated with HIV infection (Sridama *et al.*, 1982; Weinberg, 1984; Biedermann *et al.*, 1995; Hocke *et al.*, 1995). Early reports on pregnancy in HIV-infected women seemed to support the hypothesis that it accelerates disease progression. However, these studies involved small numbers of women and lacked control groups or the ability to adjust for other factors known to influence disease progression, such as disease stage or time of HIV exposure (Scott *et al.*, 1985; Koonin *et al.*, 1989; Lindgren *et al.*, 1991). Further, several studies suggest that pregnancy does not necessarily accelerate the normal rate of decline in CD4 count in HIV+ women (Nightingale *et al.*, 1992; Newell *et al.*, 1997) or increase the risk of HIV disease progression (Miotti *et al.*, 1992; Brettle *et al.*, 1995; Hocke *et al.*, 1995; Temmerman *et al.*, 1995; Bessinger *et al.*, 1998; Burns *et al.*, 1998; Weisser *et al.*, 1998; Deschamps *et al.*, 2000; Saada *et al.*, 2000). The decreasing incidence of pregnancy and by decreasing CD4 cell counts has two consequences. First, in terms of projections of the need for prevention of MTCT interventions and other HIV care for pregnant women and their children, acknowledging the fact that WHO experts now recommend specific antiretroviral regimens according to different clinical situations. Secondly, in terms of strategies for scaling-up HIV care (WHO, 2004a). If women with advanced immunosuppression are less likely to become pregnant, then women who need HAART and their families will be less likely to be recruited into prenatal care facilities than those who do not need HAART.

2.6. CD4 count and CD4 percentage in HIV-infected people

The absolute CD4 count is a measurement of the number of functional CD4 T-cells circulating in the blood. The absolute CD4 count is measured by a simple blood test and is reported as the number of CD4 cells per cubic millimeter of blood. HIV-negative people typically have absolute CD4 counts between 600 and 1200 cells per cubic millimeter; while HIV-infected persons have counts that are typically less than 500, and people with AIDS can have 200 CD4 cells per cubic millimeter or less. Hence, the lower the absolute CD4 count, the weaker the immune system.

The absolute CD4 cell count is used routinely in the evaluation and monitoring of HIV-infected people (CDC, 1989); forming a basis in developing eligibility for trials of new anti-retroviral therapies and as an endpoint measure of drug efficiency. The

absolute CD4 count is calculated from both the total and differential white blood cells count. Variability in the CD4 measurement may occur as the result of laboratory test error as well as intrapersonal temporal fluctuations due to biological factors such as diurnal variation, stress and infections (Malone *et al.*, 1990). Since certain management decisions such as initiation of prophylaxis against *Pneumocystis carinii* pneumonia (PCP), may be based on a single CD4 count, CD4 measurement error may have important clinical consequences. Hoover *et al.* (1992) have demonstrated a high probability of patients reported to have lower than the true CD4 cell count as a result of CD4 measurement error, unless confirmatory tests are done, and both these test results are below the threshold limit. In contrast, the CD4% which is measured directly on a flow cytometer is much less variable (Taylor *et al.*, 1989; Malone *et al.*, 1990; Hoover *et al.*, 1992) with coefficient of variability of 6-24% compared with 19-40% for the absolute CD4 cell count (Giorgi *et al.*, 1990).

Several studies have also shown that the CD4% (or the rate of change in CD4%) is a better predictor of clinical progression (Taylor *et al.*, 1989; Burcham *et al.*, 1991). It has therefore been suggested that the CD4% rather than the absolute cell number should be used for patient monitoring. A further advantage of using CD4% is financial, since it requires only the flow cytometer results not the white blood cell count and differential (Yu *et al.*, 1997). An important prerequisite to the more widespread adoption of CD4 measurements into clinical practice and research is a demonstration of statistical relationship between the absolute CD4 count and CD4 percentage. And also the influence on the relationships of important demographic and clinical factors, such as gender, risk group, on AIDS diagnosis and use of PCP prophylaxis (Yu *et al.*, 1997).

2.7. Factors that influence a CD4 cell count

Factors which affect CD4+ T lymphocyte counts are associated with a variety of conditions including many viral, fungal, parasitic and bacterial infection, burns, trauma, intravenous injections of foreign proteins, malnutrition, over-exercising, pregnancy, corticosteroid use, time of day variation, fatigue, smoking, psychological stress and social isolation. Cancer chemotherapy can dramatically lower the CD4 count. The low CD4 counts caused by some of these conditions often fall below 200 cells/mm³, which is the level considered to be a marker of the progression of HIV infection and AIDS, and have been called the "hallmark" of HIV infection (Balter, 1997). Infections can have a large impact on CD4 cell counts. When the body fights an infection, the number of white blood cells (lymphocytes) goes up including the CD4 cells. Acute illnesses such as pneumonia, influenza or herpes simplex virus infection can cause the CD4 count to decline temporarily. Other infections include acute pyelonephritis, abscesses, infected wounds, cellulitis, deep tissue infections, and sepsis (Williams *et al.*, 1983). Tuberculosis is a relatively common infection in people diagnosed HIV-positive, especially when compared to the general population. It is also relatively common in other people who are immunosuppressed, such as alcoholics, the homeless, intravenous drug users (IVDUs), and people who suffer from malnutrition (Beck et al., 1985). Likewise vaccinations can cause the same effects. It is best to wait a couple of weeks after the recovery from an infection or vaccination before a CD4 test is done.

Several studies over the years have looked at the effects of severe injuries or burns on CD4 counts. They found that the severity of the burns was directly correlated with depressed CD4 percentages. Patients with greater than 25% of their body covered

with 3^{rd} degree burns had the lowest percentages on admission (Antonacci *et al.* 1982). As for low CD4 in normal human pregnancy, there are reduced CD4 percentages in the 1^{st} and 2^{nd} trimester, as well as reduced CD4/CD8 ratios in the 2^{nd} trimester (Castilla *et al.* 1989).

The daily or diurnal variation in CD4 is in such a way that the count tends to be lower in the morning and higher in the evening This is because of daily fluctuations of cortisol, where the minimum CD4 levels occur between 8:00 and 10:00 a.m and maximum occur at around 10:00 p.m. Cortisol has a daily variation with maximum at about 8:00 a.m and yet cortisol causes low CD4 and total T-lymphocyte counts (Malone *et al.*, 1990). It is therefore best to have blood drawn at the same time of day for each CD4 cell test, and to use the same laboratory.

Malnutrition causes severe immunodeficiency with depletion of CD4+ T-cells and reduction of cell mediated immunity. Reduced CD4 counts were a natural physiological effect of malnutrition; and both HIV and malnutrition lead to a state of anergy with failure of cell-mediated immunity (Hegde *et al.* 1999).

Several researches have reported CD4 counts to be influenced by gender and being higher in women than men. Gender-associated viral load differences are more likely to be associated with inherent biological differences in men and women and could be due to diurnal variation (Bofill *et al.* 1992; Grinszte *et al.*, 2008). Further, sex hormone effect could be the possible explanation for the observed gender difference in CD4 counts, as the circulating lymphocytes have receptors for androgens and oestrogens (Grossman, 1985). However, according to Smith *et al.* (2004), sex, risk group, age, and HAART regimen were not associated with increases in CD4 cell counts.

2.8. CD4 count in resource limited settings

The CD4 count is a very important test in the management of patients with HIV/AIDS. There is a lot of effort in progress to increase access to antiretroviral treatment for HIV-positive patients in developing countries. However, essential laboratory tests such as the measurement of CD4 T lymphocytes in the peripheral blood remain expensive to carry out. It is no surprise that CD4 counts cannot be widely applied in resource- poor settings and hence the limited use of ART in these countries. Much as there are a number of campaigns to fight HIV/AIDS such as those instituted by WHO and Global Fund, they cannot be successful without monitoring of CD4 count, which is limited by facilities in resource limited areas. Following the increase in the number of people living with HIV to an alarming 35millions worldwide, there is massive effort to enable at least three millions of them to access life-saving antiretroviral treatment. This calls for simple and affordable methods to measure and monitor the infection using absolute CD4 counts (Bentwich, 2005). In patients with HIV infection, CD4 counts are essential for determining the clinical staging HIV-infected patients, determining their need for antiretroviral medications, monitoring the course of their infection, for evaluating the treatment outcome and changing treatment when and if necessary (Department of Health and Human Services, 2005). The CD4 count, which is expressed in adults as the absolute number of CD4 cells per microliter of blood, and in children as a percentage of total lymphocytes or total T lymphocytes; has enormous prognostic and therapeutic implications, and forms the basis for most HIV treatment decisions (O'Brien et al.,

2.9. CD4 counting methods for HIV monitoring

More than 35 million HIV-infected people live in developing countries with significant resource limitations. Although six million people living in developing countries are in urgent need of antiretroviral therapy, only 700,000 currently receive effective treatment (WHO, 2004b). Global treatment efforts, including the World Health Organization's "3 by 5" Initiative, aim at extending therapy to several million people over the next few years (WHO, 2004c). While the cost of antiretroviral medications has dropped considerably, other obstacles, including the cost, technical, and operational requirements of CD4 counts, viral loads, and other sophisticated diagnostic tests used to initiate and monitor HIV treatment, remain to be addressed (Rodriguez *et al.*, 2005). Several technologies for determining the absolute number of CD4 T-lymphocytes have been developed and evaluated in multicentric studies (WHO, 2007). The technologies are either flow cytometric or non-flow cytometric. The choice of the methods depends upon multiple factors including cost of the machine and the reagents, technical and operational know-how, among others (Janossy and Jani, 2000; Rodriguez *et al.*, 2005).

2.9.1. Flow cytometers

Flow cytometry is a technology that simultaneously measures certain cell parameters as the cells flow in a fluid stream and in single file past an analytical laser light source. It is utilized in the laboratory for measurement of CD4 counts in the evaluation and follow-up of immune deficiency (Villas, 1998). Immunofluorescence analysis by flow cytometry is the reference standard for CD4 T lymphocytes measurements and also the method of choice if a large throughput of samples is required (Zijenah et al., 2006). The flow cytometric assays work on the principle of scattering of light due to different sizes, granularity or internal complexity of the cells passing thorough the laser beam. And also by the relative fluorescence intensity emitted by the cells after staining with the specific monoclonal antibodies to cell surface markers such as CD3, CD4, and CD8 that are tagged with different fluorescence dyes is measured. The population of cells of interest can be thus identified and gated for further analysis within. The monoclonal antibodies specifically bind different surface receptors like CD4 for T helper cells (WHO 2007). Several factors including the cost of a flow cytometer (USD \$30,000 to \$150,000), not being available in the remote area, technical and operational complexity, the need for reliable electricity, and the high cost of reagents have made these instruments impractical and/or difficult to sustain in resource-scarce settings (Rodriguez et al., 2005). It seems not a proper technique for CD4 count in the developing countries such as Uganda. The urgent need for affordable and technically simple CD4 diagnostics is widely recognized (Sherman et al., 1999; Bartholomew, 2001; Huff, 2001; Glencross et al., 2002a).

Several efforts have been made to develop alternative, affordable CD4 counting methods for resource-poor settings. Single-purpose flow cytometers have been designed solely for counting CD4 cells, such as the Becton Dickinson FACSCount, the Partec CyFlow, and desktop instruments from Guava and PointCare Technologies (Rodriguez *et al.*, 2005). Although these newer versions make flow cytometry more affordable in some settings, reagent costs remain high, and the instruments remain expensive and in most cases, technically complex (Sherman *et al.*, 1999;

Bartholomew, 2001; Huff, 2001; Kannangai *et al.* 2001; Glencross *et al.*, 2002a, Glencross *et al.*, 2002b; Janossy *et al.*, 2002a, Janossy *et al.*, 2002b). Low-cost microbead separation of CD4 cells from other blood cells, followed by standard manual cell counting techniques using a light microscope, offers significantly lower reagent costs than flow cytometry. These methods, however, have low throughput, are extremely labor intensive, and appear to be less accurate than traditional flow cytometry; thus, they have not been widely adopted (Landay *et al.*, 1993; Lyamuya *et al.*, 1996; Diagbouga *et al.*, 1999; Kannangai *et al.* 2000, 2001; Didier *et al.*, 2001).

2.9.2. Pima CD4

The Pima CD4 is an automated immune hematology system which comprises of a disposable Pima test cartridge and a Pima analyzer which enables the determination of absolute counts of T-helper cells in whole blood. The disposable Pima test cartridge contains dried reagents needed to perform the test. The reagents include two different fluorescent dyes. One antibody is a mouse anti-human CD3 monoclonal antibody conjugated to phycoerythrin (PE)-Cy5. The second antibody is a mouse anti-human CD4 monoclonal antibody conjugated to Pycoerythrin PE). For example a group of ycobiliproteins can also be used to label antibodies for studying cell surface antigens. Its strong visible absorption band excites efficiently over a fairly wide range of wavelength from 440 nm to 580 nm with its emission maximum at 575 nm. On excitation E, emits orange fluorescence. The Pima analyser is a portable bench-top fixed volume cytometer used for the processing and analysis of the sample in a Pima test cartridge. The equipment consists of the Pima nalyser display screen, a key pad, 2x USB ports, cartridge slot door, power on/off and power connector ports where the transformer is connected at the back of the analyzer. The Pima analyzer is equipped
with miniaturized low-cost multi color fluorescence imaging optics. Fluorescence signals are detected by a charge coupled device (CCD) board camera and analyzed using appropriate software algorithms on board and embedded computer (WIC, 2009).

A low sample volume of approximately 25 microliter (μ L) capillary or venous whole blood is collected into the test cartridge, equipped with means to take up 5 microliters (µl) of sample and which is then capped. The Pima test cartridge is inserted into the Analyser and the sample sealed within the cartridge is processed. The test is performed in its entirety within the confinement of the cartridge and no part of the analyzer has at any time contact to the sample. This minimizes the risk of analyzer contamination and sample carry-over between measurements. After insertion of the test cartridge in the analyzer peristaltic movement first transports the sample into the incubation compartment where the sample interacts with the specific antibodies fluorescent-labeled T-helper cells with two different fluorescent dyes. After the incubation time the stained sample is transferred into the separate reading compartment of the cartridge. The Pima analyzer is equipped with miniaturized low-cost multi-color fluorescence imaging optics. Fluorescence signals are detected by a Charge Coupled Device board camera and analyzed using proprietary software algorithms on board an embedded computer. T-helper cells carry both CD3 and CD4 surface antigens and therefore emit light at wave lengths specific for both conjugates, PE and PE-Cy5. This allows the specific differentiation of T-helper cells from other blood cell types carrying only one of the two surface antigens. During the course of processing the test, data is recorded, analysed and interpreted using software embedded within the analyser. Upon completion of the test the cartridge is removed from the analyser and a test result in cells/ μ l is displayed. Results are also stored in an on-board archive.

The Pima CD4 cartridge is a unique and breakthrough technology in the enumeration of T-helper cells in whole blood samples. Designed equally to suit the needs of the healthcare professional in the field or in the laboratory environment the Pima analyzer is a more affordable, effective and valuable point-of-care diagnostic tool. It is intended to be used for the ongoing monitoring of HIV infected patients for the progression of disease and for the assessment of outcome of anti-retroviral treatment (Inverness Medical 2009)

The increasing demand for CD4+ T-cell testing, especially in resource-limited countries, means that these tests may be performed within most referral laboratory setting as well as in the simplest circumstances in the remote parts of countries with a wide range of climates (Nicholson *et al.*, 1994; Pattanapanyasat and Thakar, 2005). The tests may be performed by highly trained specialists or by healthcare workers with limited training in performing laboratory tests. Whatever the circumstances, these tests must exhibit the highest standards of performance and Pima CD4 equipment is designed to meet these needs (WIC, 2009).

2.10. Evaluating test assay precision

Precision is defined as the closeness of agreement between independent results of measurements obtained under stipulated conditions it is solely related to the random error of measurements and has no relation to trueness/accuracy (Linnet and Boyd, 2006). There are various terminologies associated with evaluation of test assay precision and these include: repeatability and reproducibility. Repeatability, also known as within-run precision, is defined as the closeness of agreement between results of successive measurements obtained under identical conditions.

Reproducibility the total or within laboratory precision refers to the closeness of agreement between results of successive measurements obtained under different conditions (time, operators, calibrators, reagents, and laboratory). While the term precision relates to the concept of variation around a central value, imprecision is actually what is measured (Chester, 2008).

Test assay precision is a quantitative measure (usually expressed as standard deviation, coefficient of variation) of the random variation between a series of measurements from multiple sampling of the same homogenous sample under the prescribed conditions. For a normal distribution, the measure of imprecision is the standard deviation (SD). The precision of a method should be tested at at-least two levels; each run in duplicate, with two runs per day over 20 days (CLSI 2004). The EP05-A2 protocol recommends that the assessment is performed on at least two levels, as precision can differ over the analytical range of an assay. One quality control (QC) sample should be at least included in each assay and the QC material used for the precision assessment should be different to that used to control the run. For purposes of this study Stabilized whole-blood samples from two CD-Check plus CD4 Low and normal (Streck, Omaha, NE) were used as control materials.

2.11. Cell gating

Cell gating is the identification of groups of similar cells of interest. Gating of data can be defined through a gate, which is a numerical or graphical boundary that can be used to define the characteristics of particles to include for further analysis (Appendix I). For example, in a blood sample containing a mixed population of cells, one might want to restrict the analysis to only the lymphocytes. The gate or region of cell populations is often made difficult by variable numbers, size, shape, and location of both target and non-target cell populations. This variability may be due to debris arising from problems with sample handling or reagents, or may be due to changes in cell populations arising from disease or specific genetic differences. In flow cytometry, different cell types are usually selected or "gated" by a series of 1- or 2dimensional geometric subsets of the measurements made on each cell. Each member of the sample corresponds to the physical properties of a biological cell - known as forward scatter (FSC) and side scatter (SSC) and antibody binding activity, through fluorescence intensity measurements (Gosink *et al.*, 2009). Gating is an integral component of the analysis of large flow cytometry data sets (Naumann *et al.*, 2010). There a number of different cell gating and these include T cell gating and, Panleucogating.

There are various T lymphocyte gating strategies based on the CD markers carried on the different cells. The CD3+ T cell employs the CD3-specific monoclonal antibodies and the number of CD4+ CD3+ T cells can be estimated once all CD3+ T cells are gated. A CD45 gating is a more reliable method for assessing lymphocyte gate purity and lymphoid cell recovery on the basis of differential CD45 marker density expression has been developed. This method uses two markers; CD45 and CD14 (CD45 is a pan-leukocyte marker expressed at different intensities on leukocytes (granulocytes CD45+; Cytotoxic CD45++; lymphocytes CD45+++ or more while the CD14 marker is selectively expressed by monocytes). This CD45+++/CD14backgating can gate all lymphocytes in the acquired events and maximize their purity by excluding unwanted non-lymphocytes (WHO 2007) The advantages of CD45 gating include easy differentiation of lymphocytes even in the presence of a large amount of debris, thus, the lysed sample can be acquired without an intermittent washing step (lyse/no-wash staining). Additionally, there is no need to use the isotype control thus saving the cost of the reagents. However, the use of CD45/CD14 gating strategy is not being used clinically; as it has been replaced by a modified one. known as Panleucogating (PLG) that uses CD45 and side scatter for gating. Panleucogating is a two-colour strategy that uses total leucocytes as the common denominator, in which total leucocytes are identified and gated by their side scatter and CD45+ characteristics. After staining with CD45 FITC and CD4-PE, leucocytes and lymphocytes are identified and gated by drawing two regions: one around all leucocytes and the other set on all bright CD45+ cells with low SSC. Lymphocytes gated in this region are further analyzed for CD4 T lymphocytes by using SSC against CD4 T lymphocytes with other fluorochrome. The CD4 T lymphocytes are easily distinguished from non-CD4+T-cells and %CD4 is then obtained as a percentage of total lymphocytes. The same analysis protocol can also be applied to CD8+ T-cells using CD45/CD8 (WHO 2007). This CD45-assisted Panleucogating technique is now widely accepted, since it is a simple, better, and cost-effective CD4 testing that is suitable in the resource-poor areas of the world. This approach can be used to test samples up to five days after collection (Glencross et al., 2002b).

The most important types of gating are: Bivariate cell-type gating, which is the identification of lymphocytes from scatter plots of forward-scatter versus side-scatter measurements; and Univariate fluorescence-channel gating which is the identification of cells that recognize a particular antibody. However, there is no cogent reason for restriction of gating to one- and two- dimensional projections of flow cytometry point clouds (Roederer and Hardy, 2001).

Data visualization and analysis often raises questions pertaining to the gating of cell populations. While manual gating is relatively robust to unanticipated cell population distributions, it suffers from the potential for operator bias and it is labor-intensive. In fact all gating methods have their drawbacks in particular cases, and tools and procedures are needed for evaluation of the results of the gating process (Gosink et al., 2009; Pynea et al., 2009). A critical bottleneck in data analysis is gating, the identification of groups of similar cells for further study. The process involves identification of regions in multivariate space containing homogeneous cell populations of interest. Generally, gating has been performed manually by expert users, but manual gating is subject to user variability, which can potentially impact results (Gratama et al., 2002; Van Blerk et al., 2003, Satoh et al., 2008). However, a number of methods have been developed to automate the gating process (Roederer and Hardy, 2001; Achuthanandam et al., 2008; Boedigheimer and Ferbas, 2008; Lo et al., 2008). These include model-based methods such as multivariate mixture models that describe the joint density of the flow cytometry data as a mixture of simpler distributions (Boedigheimer and Ferbas, 2008; Lo et al., 2008). The simplest of these methods utilizes a mixture of multivariate Gaussian distributions (Boedigheimer and Ferbas, 2008). However it is not sufficiently flexible to model the outliers or asymmetrical cell populations frequently found in flow cytometry data (Lo et al., 2008). A more recent approach compensates for these effects by applying a data transformation during the model fitting process (Sugar and James, 2003; Lo et al., 2008). This transformation makes data more symmetric, while the use of a multivariate distribution allows the model to handle outliers (McLachlan G, and Peel D, 1998; Sugar and James, 2003; Lo et al., 2008).

CHAPTER THREE: MATERIALS AND METHODS

3.1.0. Study design

This was a cross-sectional study designed to evaluate the performance of Pima CD4 in the enumeration of CD4+ T-cells in whole blood of HIV sero-positive persons. Aspects of system performance validated in this study were accuracy and precision of cell counting using capillary and venous whole blood samples, as well as the types of errors and their frequency that occurred when using the Pima CD4. Prior to the beginning of the study, the purpose of the study and its potential risks and benefits were explained to the study patients (Appendix II). Patients were enrolled in the study only after giving their written informed consent (Appendix III). Patient demographics and brief medical history were then collected and recorded.

3.1.1. Study site

The study was conducted at the Infectious Diseases Institute (IDI) Limited that houses the IDI HIV clinic and Makerere University-Johns Hopkins University laboratory. IDI is located in Mulago Hospital complex in the northern part of the city of Kampala in Kawempe Division, Uganda and is part of Makerere University College of Health Sciences. HIV positive patients (called friends at IDI) are supported with a number of services including free antiretroviral therapy, specialist consultation and a supportive clinical environment. The clinic also serves as a platform for IDI's research and training activities. The out-patient clinic at IDI has enrolled over 20,000 HIV infected patients with more than 13,000 in active follow-up and over 9,000 on first line antiretroviral treatment. CD4 is one of the routine tests that are performed on the scheduled patients every week. Thus, majority of the patients used in this study were coming from all the divisions of Kampala district. All consented patients who attended the Adult HIV clinic were recruited. Sample size was calculated using the Kish and Leslie (1965) at 95% confidence interval and prevalence of HIV infection in adults for urban areas in Uganda was estimated to be 10.1%.

 $n = Z^2 pq/I^2$

Z =1.96

Where Z = Standard deviation

- n = Sample size
- p = Estimated prevalence = 10.1% (0.101)
- q = 1 p = 1 0.101
- I = Allowable error 5% (0.05)

Therefore, the minimum n = 143 samples

In this study, two hundred and six (206) consecutive, eligible patients either initiating ART or on ART attending the clinic were enrolled between November 2009 and February 2010. This sample size was chosen to cater for eventualities such as failed runs, insufficient or clotted samples and missing data. Patients too were willing to participate in the study and reagents were readily available. In addition, a larger sample size leads to increased precision in estimates of various properties of the population.

3.1.3. Study population

The study population included men and women 18 years of age and above, with confirmed serological diagnosis of HIV infection and attending the adult HIV clinic at Infectious Diseases Institute (IDI) for HIV/AIDS treatment. HIV patients are scheduled

routinely to attend the clinic everyday from Monday to Friday between 10:00am and 12:00pm. Patients blood samples were regularly taken at the same time of the day to ensure consistency in CD4 counts.

3.1.4. Inclusion criteria of study participants

The study recruited HIV infected persons attending the adult Infectious Diseases Institute for HIV/AIDS treatment who were at least 18 years of age. These patients had serologic confirmed, documented HIV infection and agreed to complete all aspects of the study. Participants were either initiating ART or on ART and who provided written, informed consent to participate in the study. Consecutive eligible patients attending the adult HIV Clinic were approached for screening to join the study. Once a candidate for study entry was identified, study details were carefully discussed with the subject, who was asked to sign the approved consent form if he/she agreed to participate in the study.

3.1.5. Exclusion criteria of study participants

A patient was considered ineligible for the study if he/she had already participated in a previous study similar to this one and/or enrolled in a study to evaluate a new drug but was unable or unwilling to provide informed consent. Also patients were excluded if one had a blood draw on the same day but with hemoglobin level less than 8g/d or if a patient was receiving chemotherapy for a malignancy. Children were not included in this study because Infants and children are unable to give informed consent. Also there are still many challenges related to HIV diagnosis and monitoring in children (Ekpini and Gilks, 2005; ITPC. 2007; WHO, 2006, 2007; Bowen *et al.*, 2008).

3.2.0 Study materials

Materials needed for collecting venous blood samples and BD FASCalibur testing included: Vacutainers tubes with EDTA anticoagulants, vacutainer needles, cotton wool, Alcohol swabs, powder free exam gloves, calibrates, Lysing solution, Facsflow solution and Multi test reagent with Trucount tubes. Whereas materials for the Pima CD4 test included: Pima analyzers, Pima CD4 Test cartridges, lancets and pipettes.

3.3.0 Laboratory analysis (sample collection and CD4 T-cell analysis)

CD4 lymphocyte count was determined in duplicate on the same blood specimen on the same day of collection by using Pima CD4 (Inverness Medical Innovations) and by flow cytometry on a BD FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ), using the MultiTEST reagents and TruCOUNT beads, and analyzed using MultiSET software (Appendix IV).

3.2.1. Sample collection

For each consented patient, two capillary whole blood samples from the 3rd and 4th fingers of the non-dominant hand were obtained by fingerstick for immediate measurement of CD4+ T-cell count with the Pima CD4. A drop of blood was then applied directly into the end-to-end capillary attached to the Pima CD4 test cartridge according to the manufacturer's recommended procedure (Appendix V). The capillary was filled within 2-3 seconds and no further blood was drawn into the cartridge. Successful filling was visually verified via the control window on the cartridge after which the cartridge was removed from finger and closed with seal cap. Immediately after filling, the cartridge was inserted into the Pima analyzer (this step was completed within 1 minute after filling of cartridge with blood).

Venipuncture was then performed on the same participant drawing 8 ml of venous whole blood which was transferred into two 4ml K₂EDTA vacutainer tubes. For the Pima CD4 cartridge, one tube of the fresh venous K2EDTA blood was gently inverted 10-15 times to ensure proper sample mixing before withdrawing for Pima CD4 testing. Five microliters of each fresh venous blood was then applied onto two Pima CD4 test cartridge using a pipette and was immediately inserted into the Pima analyzer for testing (Appendix VI).

3.2.2 Pima CD4 analysis

Insertion of the Pima CD4 cartridge into the Pima analyzer automatically begins the testing process, providing a direct CD4 measurement within 20 minutes. Testing with Pima CD4 was performed within 1-4 hours of sample collection. The result of the Pima CD4 test was calculated automatically by the Pima analyzer. CD4+ T-cell counts were displayed on the analyzer screen and were also stored in the analyzer's memory. Pima CD4 test results were printed and the Pima CD4 printout was attached to the source document.

3.2.3 BD FACSCaliber flow cytometry

In this method, TruCount, test tubes that contain a known number of brightly fluorescent polystyrene beads were provided by the manufacturer. Fresh whole blood from the K₂EDTA tube for each patient was accurately pipetted into the tubes and mixed with fluorochrome-labeled monoclonal antibodies. The erythrocytes were lysed, and this mixture was analyzed on the flow cytometer. All samples were processed by flow cytometry according to the laboratory standard operating procedure of Makerere University-Johns Hopkins University (MU-JHU) research laboratory, as a reference method. The majority of samples were processed within 4 hrs of blood

collection, and all were processed and analyzed within 8 hrs of blood collection. For evaluation of day-to-day variability, one reference material was measured using Pima CD4 and one measurement on BD FACSCalibur were performed using CD-Chex Plus reference materials (MediMark, Grenoble Cedex 2, France). The results were compared with the specified ranges for the reference material; and if they fell out of the range, the medical laboratory technologist troubleshot the Pima CD4 system and the reference material analyzed again.

3.3.0. Quality Control (QC)

To ensure quality control of the flow cytometric immunophenotyping method with regard to the performance of both the personnel and the instrument, the same lots of reagents were used throughout the study. In addition, all the immunostaining procedures and the flow cytometric analyses were performed by the same operator for each instrument. Also, the FCM photomultiplier tube voltage, sensitivity, and fluorescent compensation settings were optimized prior to sample acquisition and analysis by using Calibrite beads which are a control set of fluorochrome-integrated beads (BD Biosciences), and reference cartridge for the FACSCaliber and Pima CD4 respectively. A vial of reference material at two different concentrations Low and Normal were run on both Pima CD4 and BD FACSCalibur as per the manufacturers' instructions. Results of reference measurements were recorded on the appropriate log sheet.

3.4.0. Accuracy of CD4 counting on the Pima CD4

The accuracy of the Pima CD4 test for enumerating CD4+ T cells was assessed by comparison with BD FACSCaliber (reference method) where venous and capillary whole blood from 206 HIV positive adult patients were collected and analyzed in

duplicate by both methods. The CD4 counts were compared using a paired t-test. Paired data was compared by linear regression, including slopes, intercepts and Pearson correlation coefficients. Analysis of agreement between the two methods was done by the Bland and Altman method (1986), which compares the difference between paired measurements against the mean of the two measurements. Then the results were presented in the form of scatter plots.

3.5.0 Assessment of precision of CD4 counting

To assess the precision of the Pima CD4 test, two fresh patient blood samples representing two diagnostic cutoffs (that are critical for medical use), 200 cells/µl and 350 cells/µl concentrations were repeatedly assayed by use of 20 replicates (CLSI 2004) for assessment of the within-run variation of the Pima CD4. For determination of between-run assay variation, stabilized whole-blood samples from two CD-Chex® Plus CD4 Low and normal (MediMark, Grenoble Cedex 2, France), a stable whole-blood control with assigned CD4 values were used. The two samples were analyzed by running 20 replicates for a period of twenty days on the Pima CD4 instrument. Within-run variation and between-run variation mean; standard deviation; and coefficient of variation (CV) were calculated to verify precision recommendations by the manufacturer. The CD4 measurements of the 20 replicates from patient sample 1 and 2 for within-run assay variation and results of CD4 counts from the low and normal controls for between-run were plotted on a radar graph for a quick visual interpretation of the relationship between the replicate results.

3.6.0 Types of errors and their frequencies

When an error occurred, the test would be aborted automatically, the cartridge ejected and the Pima analyzer would display an error code on the screen. These errors were compiled by the operator by recording the number and type of each error observed on the Pima CD4 test. And to determine the errors and their frequencies, data on errors committed by operator when using Pima CD4, were analyzed using descriptive statistics (frequencies %).

3.7.0. Ethical approval

The study was reviewed and approved by the Infectious Diseases Institute (IDI) Clinic Cohort Committee and the Uganda National Council for Science and Technology (Appendix VII)

3.8.0. Statistical analysis

Statistical analyses were performed using SAS software (SAS Institute Inc., 2008). The demographic characteristics of the study population were analysed using Chi square. Pima CD4 test results (using venous whole blood and capillary blood) were compared with those obtained by BD FACSCaliber (using venous whole blood). The CD4 counts from Pima CD4 and BD FASCaliber were compared using a paired t-test (at P=0.05 level). Paired data was compared by linear regression, including slopes, intercepts and Pearson correlation coefficients. Analysis of agreement between the two methods was done by the Bland and Altman method (1986), which compares the difference between paired measurements against the mean of the two measurements. According to Bland and Altman, limits of agreement summarize lack of agreement by calculating bias, which is estimated by the mean difference (D) and the standard

deviation of the differences (S). If the differences are normally distributed, then 95% of differences will lie between D - $1.96 \times S$ and D + $1.96 \times S$. Data on errors committed by operator when using Pima CD4, was analyzed using descriptive statistics (frequencies %).

CHAPTER FOUR: RESULTS

4.1. Characteristics of the study population

The study was conducted over a period of nine months from September 2009 to May 2010 and a total of 206 HIV-positive individuals were enrolled. Considering gender difference, out of the 206 HIV-infected individuals, 156 (75.7%) were females and 50 (24.3%) were males (Table 1). Overall, the majority of the patients were in age group 31-50 years (63.5% of females and 80.0% of males). Mean age was 34.9 ± 0.6 and 37.9 ± 0.4 years for females and males respectively and age range was 18-68 years and 20-55 years for females and males respectively (Table 1). By age range, 85.5% of the patients were females and 14.6% were males and were highly significantly different (P<0.0001) for patients aged 30 years and below. For patients in age range 31-50 years, 71.2% were females and 28.8% were males and were highly significantly different (P<0.0001). For patients above 50 years of age, 83.3% were females and 16.7% were males and were highly significantly different (P=0.0209)

Age group (years)	Female N (%)	Male N (%)	P-value	Total N (%)	
≤30	47(85.5)	8(14.5)	< 0.0001	55 (26.7)	
31-50	99(71.2)	40(28.8)	< 0.0001	139 (67.5)	
>50	10(83.3)	2(16.7)	< 0.0209	12 (5.8)	
Total	156 (75.7%)	50 (24.3%)	< 0.0001	206 (100)	
Mean Age, yrs± SD	34.9±0.6	37.9±0.4		35.6±9.7	

Table 1:Distribution of the study patients by age and gender

4.2. CD4 counts for the various age groups of the patients by gender using Pima CD4 for venous blood

Using Pima CD4 for venous whole blood, the mean CD4 counts in age group \leq 30 years were higher in females (424.4±231.2 cells/µL) than in males (216.7±89.0 cells/µL) and were significantly different ($|t_{df=62}| = 2.92$, P=0.049). The mean CD4 counts in age group 31-50 years were higher in females (394.8±188.1 cells/µL) than in males (381.0±191.3 cells/µL) and were not significantly different ($|t_{df=128}| = 0.38$, P=0.7069). Further, the mean CD4 counts in age group >50 years were higher in females (421.4±221.7 cells/µL) than in males (265.0±4.2 cells/µL) and were significantly different ($|t_{df=10}| = 2.23$, P=0.0297; Fig. 1).



Figure 1 Mean CD4 counts distribution in age groups for Pima CD4 using venous blood

4.3. CD4 counts in pregnant and non-pregnant female patients

Out of the 156 female patients, 10.3% (16) were pregnant and 89.7% (140) were not pregnant. When using BD FASCaliber for venous whole blood, the mean CD4 counts were significantly lower (P=0.0007) for pregnant (258 ± 111 cells/µL) than for the non-pregnant (460 ± 283 cells/µl) women.



Figure 2 CD4 counts in pregnant and non-pregnant female patients by the different testing instruments and blood source

Similarly, the mean CD4 counts were lower for pregnant ($254\pm120 \text{ cells/}\mu\text{L}$) than for the non-pregnant ($424\pm206 \text{ cells/}\mu\text{L}$) women and were significantly different ($|t_{df=154}|$ = 3.25, P=0.0014) when using Pima CD4 for venous whole blood. Also, the mean CD4 counts were lower for pregnant ($228\pm105 \text{ cells/}\mu\text{L}$) than for the non-pregnant ($402\pm185 \text{ cells/}\mu\text{L}$) women and were significantly different ($|t_{df=154}|$ = 3.68, P=0.0003) when using Pima CD4 for capillary blood (Fig. 2; Table 2). The mean CD4 count for BD FASCaliber using venous blood and Pima CD4 using both venous and capillary blood for pregnant women were not significantly different (P=0.7202).

Table 2CD4 counts in pregnant and non-pregnant female patients by the
different testing instruments and blood source

Instrument (type of blood	Mean CD4 counts \pm SD (cells/µl)		t statistic	p value
and source of blood)	Pregnant	Non pregnant		
BD FACSCaliber venous	258±111	460±283	3.48	< 0.0007
PIMA CD4 venous	254±120	424±206	3.25	< 0.0014
PIMA CD4 capillary	228±105	402±185	3.68	< 0.0003

4.4.1 Comparison of results using BD FACSCaliber and Pima CD4 on venous whole and capillary blood

Using venous whole blood, the range of CD4 counts was 18 - 1500 and 14 - 1250 cells/µl for BD FACSCaliber and Pima CD4 respectively. Using capillary blood, the range of CD4 count was 10 - 936 cells/µL. Out of the 206 venous whole blood samples analyzed by BD FACSCaliber, 27 (13.1%) revealed CD4 counts below 200 cells/µL, 62 (30.1%) between 201 and 350, 53 (25.7%) between 351 and 500, 49 (23.8%) between 501 and 750, and 15 (7.3%) above 750 cells/µL. Out of the 206 venous whole blood samples analyzed by Pima CD4, 28 (13.6%) revealed CD4 counts <200 cells/µL, 73 (35.4%) between 201 and 350, 51 (24.8%) between 351 and 500, 42 (20.4%) between 501 and 750, and 12 (5.8%) above 750 cells/µL. Further, out of the 206 capillary blood samples analyzed by Pima CD4, 35 (17.0%) revealed CD4 counts <200 cells/µl, 72 (35.0%) between 201 and 350, 52 (25.2%) between 351 and 500, 39 (18.9%) between 501 and 750, and 8 (3.9%) above 750 cells/µL (Fig. 3).



Figure 3 Comparison of results using BD FACSCaliber using and Pima CD4 on venous and capillary blood for the various CD4 count ranges

The mean CD4 counts in the various CD4 count ranges for BD FACSCaliber using venous whole blood and Pima CD4 using venous whole and capillary blood are summarized in Fig 4. The mean CD4 counts were 144.8±44.8, 131.3±49.2 and 133.3±47.9 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.5216) for CD4 range of ≤ 200 cells/µL.



Figure 4 Agreement of mean CD4 counts for BD FACSCaliber using venous whole blood and Pima CD4 using venous whole and capillary blood for the various CD4 count ranges

For CD4 range of 201-350 cells/µL, the mean CD4 counts were 285.7±42.0, 275.8±42.1 and 276.9±41.0 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and according to Analysis of Variance (ANOVA) test, they were not significantly different (P=0.3362). For CD4 range of 351-500 cells/µL, the mean CD4 counts were 415.5 ± 41.8 , 416.5 ± 45.0 and 423.3 ± 44.3 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.6604). For CD4 range of 501-750 cells/µL, the mean CD4 counts were 589.1±66.1, 582.2±60.9 and 590±72.4 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.8198). For CD4 range of >750 cells/µL, the mean CD4 counts were 959.2±199.5, 910.8±133.7 and 852.5±61.9 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.3035). The overall mean CD4 counts were 422±220 cells/µL for BD FACSCaliber using venous blood, 391±201 cells/µL for Pima CD4 using venous blood and 371±185 and were significantly different from each other (P=0.0374) (Figure 4; Table 3).

Range	BD FACSCali	ber	Pima CD4 (veno	us blood)	Pima CD4	(capillary	
(cells/µl)	(venous)				blood)		
	Mean±SD	Ν	Mean±SD	Ν	Mean±SD	N	P value
	CD4 counts		CD4 counts		CD4 counts		
	(cells/µl)		(cells/µl)		(cells/µl)		
≤200	145±45	27	131±49	28	133±48	35	0.5216
201-350	286±42	62	276±42	73	277±41	72	0.3362
351-500	417±42	53	407 <u>±</u> 45	51	423±44	52	0.6604
501-750	589±66	49	582±61	42	591±72	39	0.8198
>750	959±200	15	911±134	12	852±62	8	0.3035
Overall	422±220	206	391±201		371±185		0.0374

Table 3Agreement of mean CD4 counts between Pima CD4 and BD
FACSCaliber for the CD4 ranges

Further, the mean CD4 counts in the various age group ranges of the patients for BD FACSCaliber using venous whole blood and Pima CD4 using venous whole and capillary blood are summarized in Fig 5. The mean CD4 counts were 420.6 ± 235.9 , 388.7 ± 227.2 and 365.9 ± 191.9 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.3685) for patient who were 30 years and below. For age range 31-50 years, the mean CD4 counts were 420.7 ± 209.0 , 390.9 ± 188.4 and 375.0 ± 181.6 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.1557). For age range >50 years, the mean CD4 counts were 445.1 ± 264.2 , 395.3 ± 209.6 and 358.8 ± 200.2 cells/µL for BD FACSCaliber (venous whole blood) and Pima CD4 (capillary blood) respectively and were not significantly different (P=0.4557).



Figure 5 Agreement of mean CD4 counts for BD FACSCaliber using venous whole blood and Pima CD4 using venous and capillary whole blood for the various age groups

4.4.1. Linear regression techniques for BD FASCaliber and Pima CD4

Linear regression analysis showed that BD FACSCaliber and Pima CD4 when using venous blood were highly correlated. Linear regression slope 0.8827, intercept of 17.88 and $r^2 = 0.93$ (Fig. 6) with Pearson correlation coefficient of 0.96 (P=<0.0001) were observed. The solid line represents regression line, Pima CD4 = 0.8827 + 17.88; $r^2 = 0.93$. Pearson correlation coefficient for samples below 200 cells/µl was 0.99 (P=<0.0001).



Figure 6 Correlation analysis of BD FACSCaliber and Pima CD4 using venous blood for CD4 cell counts (cells/µl.)

Further, linear regression analysis showed BD FACSCaliber using venous blood and Pima CD4 using capillary blood were highly correlated. Linear regression slope 0.7583, intercept of 51.18 and $r^2 = 0.81$ (Fig. 7) with Pearson correlation coefficient of 0.90 (P<0.0001) were observed. The solid line represents regression line, Pima CD4 = 0.7583 + 51.18; $r^2 = 0.93$. Pearson correlation coefficient for samples < 200 cells/µL was 0.83 (P<0.0001).



Figure 7 Correlation analysis of capillary blood using Pima CD4 with whole blood obtained by BD FACSCaliber

Linear regression analysis showed that both the capillary blood and venous whole blood measurements by Pima CD4 highly correlated. Linear regression slope 1.01, intercept of 15.42 and $r^2 = 0.86$ (Fig.8) with Pearson correlation coefficient of 0.93 (P<0.0001). The solid line represents regression line, Pima CD4 = 1.01 + 15.42; r² = 0.93. Pearson correlation for CD4 counts below 200 cells/µl was 0.92 (P<0.0001).



Figure 8 Correlation analysis of Pima CD4 using capillary and venous whole blood

4.4.2. Bland-Altman techniques for BD FASCaliber and Pima CD4

According to Bland and Altman, limits of agreement summarize lack of agreement by calculating bias, which is estimated by the mean difference (D) and the standard deviation of the differences (S). If the differences are normally distributed, then 95% of differences will lie between D - $1.96 \times S$ and D + $1.96 \times S$. Bland-Altman analysis showed a bias of -31.6 CD4 cells/µL, (95% CI -39.7, +31.3) between Pima CD4 and BD FACSCaliber machines. The 95% limits of agreement were -146 CD4 cells/µL (95% CI -160, -132.5) and 83.0 (95% CI +69.2, +96.7) (Fig. 9). The pairwise comparison of accuracy by Bland-Altman analysis shows that the machines were different ($|t_{df=205}| = 7.77$, P<0.0001).



Figure 9 Bland-Altman plot comparing the mean and the difference between standard BD FACSCaliber and Pima CD4 cell counts using venous whole blood

Bland-Altman analysis showed a bias of -50.6 CD4 cells/ μ L (95% CI -64.0, -37.7) between Pima CD4 and BD FACSCaliber machines. The 95% limits of agreement were -248.7 CD4 cells/ μ L (95% CI -261.2, -216.1) and 137.0 (95% CI +114.5, +159.6) (Fig.10). The pairwise comparison by Bland-Altman analysis shows that the machines were different ($|t_{df=205}| = 7.61$, P<0.0001).



Figure 10 Bland-Altman plot comparing the mean and the difference of capillary blood using Pima CD4 with whole blood obtained by BD FACSCaliber

Bland-Altman analysis showed a bias of -19.2 CD4 cells/ μ L (95% CI -29.5, -8.9) between measurements of CD4 cells in capillary blood and venous whole blood. The 95% limits of agreement were -165.9 CD4 cells/ μ L (95% CI -183.5, -148.3) and 127.5 (95% CI +109.9, +145.1) (Fig. 11). The pairwise comparison by Bland-Altman analysis shows that the measurements were different ($|t_{df=205}| = 3.63$, P=0.0003).



Figure 11 Bland-Altman plot comparing the mean and difference of Pima CD4 using capillary and venous whole blood

4.3 Precision of T-cell counts obtained by using the Pima CD4 test on whole blood samples

Results for within-run and between-run tests for Pima CD4 are presented in table 1. For within run precision (repeatability), the mean for the low CD4 counts was 234.4 \pm 5.85 with a Coefficient of variation (CV) of 2.50% and mean for the normal CD4 counts was 483.5 \pm 8.54 with a CV of 1.77% (Table 4). For between run precision (reproducibility), the mean CD4 counts for the low CD4 counts was 239.4 \pm 6.79 with a Coefficient of variation (CV) of 2.84% and mean CD4 counts for the normal CD4 counts was 483.9 \pm 9.56 with a CV of 1.98%. The manufacturer's performance CV% claim was 5.00 for sample 1 and 2 for within-run, 9.54 and 7.05 for low and normal CD4 counts for between run respectively (Table 4).

	Within run precision		Between run precision	
	Sample 1	Sample 2	Low	Normal
Ν	20	20	20	20
Mean	234.4	483.5	239.4	483.9
Standard deviation	5.85	8.54	6.79	9.56
Coefficient of variation	2.50	1.77	2.84	1.98
Manufacturer's (CV %)	5.00	5.00	9.54	7.05

Table 4Summary for precision of Pima CD4

The Coefficient of Variations (CV's) for sample 1 and sample 2 were 2.50 and 1.77 respectively and were less than the manufacturer's recommended CV (5.00) and thus clinically acceptable. Similarly, the CV's for low and normal CD4 counts were 2.84 and 1.98 respectively and less (2.84) than the manufacturer's recommended CV's (9.54 and 7.05 respectively) and thus clinically acceptable.

Results for within-run precision of CD4 counts obtained by using Pima CD4 for sample 1 and 2 and between run precision of CD4 counts obtained by using Pima CD4 for low and normal samples together with the "ideal values" for the controls (purple-ideal lower and red-ideal upper) are presented in the precision radar plots (Fig 12A, B) below. The CD4 count measurements obtained by using Pima CD4 were precisely running (that's the 2 data points on a spoke are close to each other) along the lower and upper ideal values (green and blue respectively). Each spoke or radial line represents a replicate CD4 result, and each radial line contains two CD4 values, the "ideal" CD4 value and the Pima CD4 measurement value. There are 20 replicates, and thus 20 "spokes". In a radar plot it is not the slope that is important, but the distance between the 2 dots on the same spoke. If the 2 dots are far apart, the change is large.



Figure 12 Within-run (Plot A) and Between-run (Plot B) precision for Pima CD4 results for sample 1 and 2 and Normal and Low CD4 count samples respectively

4.5 Types and frequency of errors incurred by operators when using Pima CD4

When using venous whole blood out of the total runs (206), the operator committed 50 errors (24.3%). Of the 50 errors committed, the most prominent were channel filling (36.0%), reagent quality control (24.0%), exposure control (18.0%) and image (10.0%) Using capillary blood, the operator committed 28 (13.6%) errors out of the 206 runs. Of the 28 errors committed, the most prominent were image (39.3%), reagent quality control (28.6%), focus control (10.7%) and homogeneity (10.7%) (Fig.13).



Figure 13 Types and frequency (%) of errors committed by operator when using Pima CD4 venous and capillary blood

CHAPTER FIVE: DISCUSSION

Answering the questions posed in the introduction, the current study was conducted in a designated hospital clinic to assess accuracy, precision of CD4 counts using capillary finger stick and fresh venous whole blood and establishing the types and frequency of errors incurred when using Pima CD4. This study shows that the enumeration of CD4+ T-lymphocytes using Pima CD4 performed well when compared with the reference bead-based method and the results from the two machines were highly correlated. The CD4 counts enumerated by Pima CD4 using capillary blood was in close agreement with those in venous whole blood. Thus, rapid tests for CD4 counts can be performed on finger-prick blood samples instead of venous blood when dealing with small volumes of blood, in children or subjects with difficult veins to locate. Further, within run and between run precision demonstrated that the inherent imprecision of the Pima CD4 instrument is within the manufacturer's claims. Also, results show that pregnant women had lower CD4 counts than non pregnant and female patients had a higher CD4 counts than males. Using venous and capillary whole blood the most common errors found were channel filling, image and reagent control. Despite the few short comings and due to lower cost, simplicity, low daily throughput comparing to the flow cytometry method, Pima CD4 maybe favoured as a suitable alternative for health centers and remote areas with limited access to CD4 testing centers.

It is of paramount importance that any instrument introduced for clinical purposes must first be thoroughly evaluated and shown to be giving results which are accurate. Several alternative methods to monitor CD4+ T-cell counts in HIV-infected individuals have been evaluated recently. Many of them show excellent correlation with the standard flow cytometric assays and have been successfully implemented in resource-poor settings (Didier *et al.*, 2001; Balakrishnan *et al.*, 2006; Spacek *et al.*, 2006; Lutwama *et al.*, 2008; Inverness Medical group, 2009; Glencross *et al.*, 2010). The results showed that Pima CD4 is able to count CD4 lymphocytes in HIV-infected people, but it generally underestimated CD4 lymphocytes counts. Using 206 fresh venous whole blood samples from a designated hospital clinic, a mean CD4 count of 391 ± 201 cells/µL was observed when using Pima CD4 compared to a mean of 422 ± 220 cells/µL by the BD FACSCaliber is used but these means were not significantly different ($|t_{df=410}| = 1.52$, P=0.1289). In this study the BD FACSCaliber which is a bead-based TruCOUNT method showed higher absolute CD4⁺ T-lymphocyte values compared with those obtained by the Pima CD4. A similar bias was also observed when the TruCount bead-based system was compared with the Conventional methods for counting CD4 cells, indicating that this tendency of the TruCOUNT system to provide higher CD4 counts than other methods may be a reproducible characteristic of the TruCOUNT bead-based technology (Nicholson *et al.*, 1997)

Furthermore, linear regression analysis showed that both machines were highly correlated. Linear regression $r^2 = 0.93$ with a slope of 0.8827 and intercept of 17.88, with Pearson correlation coefficient of 0.96 (P=<0.0001) were observed. These results are in agreement with earlier studies which compared performance of Pima CD4 with standard methods (flow cytometer). For example, using 149 samples in a laboratory setting, a study conducted in Germany to compare performance characteristics of Pima CD4 and BD FACSCaliber, a slope of 0.95 (0.91 to 0.99) with an intercept (95% CI) of 24 (8.5 to 37) and Pearson correlation coefficient (95% CI) of 0.96 (0.94 to 0.97) between the two measurements were obtained (Inverness Medical group,

2009). Likewise, using 50 samples in a study conducted in South Africa by Glencross *et al.* (2010) to compare the performance of four parallel Pima instruments with flow cytometric based PLG/CD4 testing as the reference standard (Beckman Coulter XL flow cytometer), a regression slope of 0.92 and Spearman correlation of 0.98 were observed.

Bland-Altman analysis showed a bias of -31.6 CD4 cells/µL (95% CI -39.7, +31.3), between both the Pima CD4 and the BD FACSCaliber machines. The 95% limits of agreement were -146 CD4 cells/µL (95% CI -160, -132.5) and 83.0 cells/µL (95% CI +69.2, +96.7). This indicates that Pima CD4 results may be as many as 146 CD4 cells/µL greater or 83.0 cells/µL lesser than BD FACSCaliber results. The results showed that the range between these two limits was generally small. The smaller the range the better the agreement between the two instruments (Bland and Altman, 1986). Results obtained by a new laboratory test that are different do not necessarily mean that the new test is in error. The true tests of any laboratory assay are its medical relevance and how well it performs in helping users make diagnostic medical decisions (Nicholson et al., 1997). Also according to Lutwama et al. (2008), for clinical purposes, this amount of variation may be acceptable so far, as the Pima CD4 methods are less expensive than flow cytometry and the limited infrastructure in remote settings may prevent the use of more accurate methods. However, the pairwise comparison of accuracy of Pima CD4 and BD FACSCaliber technologies by Bland-Altman analysis was highly significant ($|t_{df=205}| = 7.77$, P<0.0001). These results contradict other studies which have compared performance of Pima CD4 count with standard methods (flow cytometer). For example, in a study to compare performance of Pima CD4 and BD FACSCaliber in Germany, Inverness Medical group (2009)

reported a mean bias (95% CI) across all 149 samples of -10 (-22 to 3) cells/ μ L and it was not significantly different. Similarly, in South Africa, Glencross *et al.* (2010) noted an overall bias (n=50samples) of -12.02±38.7, with a bias of 2.63±13.9 for samples with a CD4 count <200 cells/ μ L (cut-off for ART in South Africa) and -19.12±44.6 for CD4 count >200 cells/ μ L. None of these differences were significant or changed decision making for ART initiation.

The difference in results could have been probably due to the sample size used in both studies. Glencross *et al.* (2010) used 50 samples, Germany; Inverness Medical group (2009) used 149 samples as compared to 206 samples used in this study. Thus, the biases between the different instruments used as reference standards though may not be great, reinforce the theory that identification of lymphocytes varies by instrument and method of lymphocyte identification. Whereas PLG/CD4 XL flow cytometer can analyze samples that have spent a few days after collection, the FACSCaliber can only analyze fresh or stabilized samples (Bentley *et al.* 1993). Differences between the studies may also have been due partially to differences in geographical location and study population characteristics such as age, race/ethnicity, and prevalence of underlying diseases, immunological and genetic factors, all of which have been shown to be associated with variations in CD4 cell counts. African populations typically have lower CD4 lymphocyte counts than their western European and Caucasian counterparts (Messele *et al.*, 1999; Clerici *et al.*, 2000)

Furthermore, the most probable explanations could have been in part due to the technical errors that might have occurred during sample preparation and/or gating of cell population. While manual gating is relatively robust to unanticipated cell
population distributions, it suffers from the potential for operator bias and it is laborintensive. In fact all gating methods have their drawbacks in particular cases, and tools and procedures are needed for evaluation of the results of the gating process (Gosink *et al.*, 2009; Pynea *et al.*, 2009). A critical bottleneck in data analysis is gating, the identification of groups of similar cells for further study. The process involves identification of regions in multivariate space containing homogeneous cell populations of interest. Generally, gating has been performed manually by expert users, but manual gating is subject to user variability, which can potentially impact results (Gratama *et al.*, 2002; Van Blerk *et al.*, 2003, Satoh *et al.*, 2008).

In this study, both machines registered gender differences in CD4 lymphocyte counts. BD FACSCaliber registered a mean CD4 count of 439 ± 227 and 368 ± 168 cells/µL for females and males respectively and were significantly different ($|t_{df=204}| = 2.00$, P=0.0464). Similarly, Pima CD4 registered a mean CD4 count of 407 ± 205 and 340 ± 184 cells/µL for females and males respectively and were also significantly different ($|t_{df=204}| = 2.04$, P=0.0423). These results generally agree with other studies in which different technologies for enumeration of CD4 lymphocytes have reported higher CD4 counts in both HIV-negative and HIV-positive women more than men (Maini *et al.*, 1996; Delmas *et al.*, 1997; Farzadegan *et al.*, 1998; Sterling *et al.*, 1998; Prins *et al.*, 1999). According to Grinszte *et al.* (2008), gender-associated viral load differences are more likely to be associated with inherent biological differences in men and women than factors linked to geographic location including race, coinfections, culture or environment. Grossman (1985) suggested that sex hormone effect could be the possible explanation for the observed gender difference in CD4 counts, as the circulating lymphocytes have receptors for androgens and oestrogens. Bofill *et al.* (1992) also suggested that the gender difference could be due to diurnal variation.

However, the main question is whether the gender differences in CD4 lymphocyte counts have implications for patient management. This is especially with regard to initiation of antiretroviral therapy since guidelines include CD4 cell counts as criteria for starting therapy (Prins et al., 1999). Before highly active antiretroviral therapy (HAART) became available, a CD4 count of 200cells/µL was regarded as the level below which antiretroviral therapy and PCP prophylaxis should be started. Due to gender differences in CD4 counts, women started ART's later than men. Nevertheless this did not have a significant difference on disease progression (Prins et al., 1999). However, since the more powerful HAART, which is initiated much earlier in infection when the CD4 cell count drops below 500cells/µL, became generally available in 1996, the situation became different (Farzadegan et al., 1998; Sterling et al., 1998; Prins et al., 1999). The gender differences in CD4 lymphocyte counts suggest a delay of initiation of therapy in women compared with men, which if it unfavorably influences disease progression may require revision of treatment guidelines to enable women to benefit equally from HAART (Prins et al., 1999; Mocroft et al., 2000).

When capillary blood was used for enumeration of lymphocytes using Pima CD4, the mean CD4 count was 371 ± 185 cells/µL and was significantly different ($|t_{df=410}| = 2.54$, P=0.0116) from BD FACSCaliber (422 ± 220 cells/µL). However, linear regression analysis showed that results from both machines were highly correlated. Linear regression slope 0.7583, intercept of 51.18 and $r^2 = 0.81$ with Pearson correlation

coefficient of 0.90 (P<0.0001) were observed. These results are in agreement with earlier studies conducted in Germany to compare performance of Pima CD4 and BD FACSCaliber by Inverness Medical group (2009). In their study, the performance of Pima CD4 with capillary whole blood samples was shown to be comparable to that with venous blood. Compared to BD FACSCalibur venous blood results, regression analysis showed a slope (95% CI) of 0.85 (0.76 to 0.94) with an intercept (95% CI) of 46.42 (-5.92 to 98.76). The Pearson correlation coefficient (95% CI) between the two measurements was 0.94 (0.89 to 0.97).

Rapid tests for CD4 counts are currently under development and could increase the availability of CD4 counting. Such tests could be performed on finger-prick blood samples, enabling them to be conducted where trained phlebotomists are not available or when difficulties are encountered with venous sampling. Finger-prick blood samples are commonly used in resource-poor settings for rapid HIV tests and malaria parasite slides and so may be more acceptable than venipuncture. However, studies comparing CD4 counts in finger-prick and venous blood are limiting (MacLennan et al., 2007). In this study, the mean CD4 counts enumerated by Pima CD4 in venous whole blood (391 ± 201 cells/µL) were higher than that in capillary blood (371 ± 185 cells/ μ L) but there was no significant difference (($|t_{df=410}| = 1.01, P=0.3142$). On the contrary, other studies have reported higher CD4 counts in capillary (skin puncture) blood than in venous whole blood but with no significant differences (Yang et al., 2001; Daae et al., 1988; MacLennan et al., 2007). This variation across studies may be partially contributed by different experimental conditions and/or sampling, systematic errors and maybe the different study populations. CD4 counts vary across populations due to a variety of demographic, environmental, immunological and genetic factors that probably persist throughout the course of HIV infection. Also previous studies have demonstrated significant diurnal variation in lymphocyte levels, and such variation may have biased the CD4 measurement (Messele *et al.*, 1999; Clerici *et al.*, 2000)

Linear regression analysis showed that both capillary and venous measurements were highly correlated. Linear regression slope 1.01, intercept of 15.42 and $r^2 = 0.86$ (Fig.10) with Pearson correlation coefficient of 0.93 (P=<0.0001). This agrees with Yang et al. (2001), Daae et al. (1988) and MacLennan et al. (2007) who reported a relation between CD4 counts in venous and capillary blood. Bland-Altman analysis showed a bias of -19.2 CD4 cells/µL (95% CI -29.5, -8.9) between measurements of CD4 cells in capillary blood and venous whole blood. The 95% limits of agreement were -165.9 CD4 cells/µL (95% CI -183.5, -148.3) and 127.5 (95% CI +109.9, +145.1) (Fig. 11). The pairwise comparison by Bland-Altman analysis shows that the measurements were significantly different ($|t_{df=205}| = 3.63$, P=0.0003). In contrast, MacLennan et al. (2007) reported an agreement between paired CD4 counts with little bias and narrow agreement. The difference in results could have been due to lack of an adapter on the channel filling tube of the cartridge of Pima CD4. In addition, HIV patients might defer in nutritional status, stage of disease presentation, medical care and behavioral practices which may have influenced the results. Finger-prick CD4 values were higher than venous CD4 counts by an average of 6.6 cells/µL (95%CI 1.0, 12.0), with limits of agreement -50.7 cells/µl (95%CI -60.3, -41.2) and 63.7 cells/µl (95% CI 54.2, 73.3). Thus, these data indicate that provided careful sampling technique is followed, finger prick blood samples could be used in place of venous blood samples in HIV-infected adults for absolute CD4 lymphocyte counts as also

recommended by MacLennan *et al.* (2007). Collection of blood by skin puncture provides a rapid and simple alternative to venipuncture when small volumes of blood are required, and is useful when dealing with children and subjects with difficult veins to locate (Cracknell *et al.*, 1995). This will potentially increase the accessibility for CD4 counting in resource-poor settings, especially once rapid tests become widely available (MacLennan *et al.*, 2007).

The mean CD4 counts were lower in pregnant than in non-pregnant female patients with a mean \pm SD CD4 counts of 258 \pm 111, 254 \pm 120 and 228 \pm 105 cells/µL in pregnant and 460±283, 424±206 and 402±185 cells/µL in non-pregnant by BD FASCaliber for venous whole blood, Pima CD4 for venous whole blood and Pima CD4 for capillary blood respectively. These results were consistent with findings of other studies that examined the effect of pregnancy on CD4+-cell counts in HIVpositive women (Burns et al., 1996; Danisman et al., 2001). Similarly, a decline in CD4 cell counts has been reported during pregnancy in HIV-negative women (Bisalinkumi et al., 1992; Dayama et al., 2003; Aina et al., 2005; Chama et al., 2009). Pregnancy has been shown to be associated with altered immunity in both laboratory and clinical studies (Sridama et al., 1982; Weinberg, 1984; Biedermann et al., 1995), which could enhance the immunosupression associated with HIV infection (Weinberg, 1984; Hocke et al., 1995). At present, the type and number of antiretroviral drugs needed, together with the foreseeable antiretroviral treatment duration partly depend on the mother's CD4 cell count (WHO, 2004a). While the MTCT risk is higher with the increasing immunosuppression of the mother (Leroy et al., 2002), whether a pregnant woman meets the criteria for receiving highly active

antiretroviral therapy (HAART) largely depends on her immunological status (WHO, 2004a).

The aim of assessing precision was to estimate the random and systematic error associated with performing the CD4 counts on Pima CD4 and also to determine the variability of repeat measurements as compared with data published by the manufacturer. In this study, within run precision demonstrated that inherent imprecision of the Pima CD4 instrument is within the manufacturer's specifications and clinically acceptable limits (Inverness Medical group 2009). A mean CD4 count for sample 1(fresh sample low CD4 count) was 234.4±5.85 with a Coefficient of Variation (CV) of 2.50% and mean CD4 count for the sample 2(fresh sample Normal CD4 count)) was 483.5±8.54 with a CV of 1.77%. Between run precision demonstrated that the overall imprecision of Pima CD4 instrument inherent and due to other external variants is within manufacturer's specifications and clinically acceptable limits (Inverness Medical group 2009). The mean for the low CD4 counts was 239.4±6.79 with a Coefficient of variation (CV) of 2.84% and mean for the normal CD4 counts was 483.9±9.56 with a CV of 1.98%. The manufacture's performance CV for Pima CD4 is 5.00% hence; these findings are within acceptable values. The study results demonstrated that the Pima CD4 maybe a reliable instrument in our setting: it is precise when operated as per manufacturer's instructions and the agreement between the Pima CD4 and the FACSCaliber is high. The underestimation of the CD4 count as suggested by the confidence intervals around the mean difference does not have clinical implications (Teav et al., 2004). In this study, within-run and between run precision of T-cell counts obtained by using the Pima CD4 for low and normal CD4 count samples were plotted together with the "ideal values" for the controls on radar plots (Fig 14A, B) to demonstrate how precise the results were. These results agree with earlier studies by various researchers who have compared new technologies with reference standard for enumerating absolute lymphocyte counts and observed low CV's (Nicholson *et al.*, 1994; Teav *et al.*, 2004; Imade *et al.*, 2005; Kannangai *et al.*, 2005; Denny *et al.*, 2008). Modern instruments have a CV of 3- 5% and when all other parameters are equal, the lower the CV, the better the test (USAID, 2008).

Pima CD4 being a relatively new innovation in Uganda, operators are bound to make mistakes when using it. In this particular study, the operator committed 24.3% errors when using venous whole blood with channel filling, reagent quality control, exposure control and image as the most prominent ones (Fig. 13). Using capillary blood, the operator committed 13.6% errors with image, reagent quality control, focus control and homogeneity as the most prominent errors (Fig. 13). In view of increasing attention focused on patient safety and the need to reduce Pima CD4 errors, it was important to collect statistics on error occurrence rates over the whole testing cycle. These errors are helpful in identifying what part of the instrument or the application is causing the problem in order for appropriate troubleshooting steps to be made. Children were not included in this study because Pima CD4 is not yet able to provide percentages and yet absolute CD4 counts are not preferred for children under 12 years old because absolute CD4 counts vary a lot. Children are monitored by CD4 percentages which is sometimes more stable indication of whether there has been a change in the immune system.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The CD4 counts of female patients were higher than that of the male patients and pregnant women patients had significantly lower CD4 counts than those for nonpregnant irrespective of the machine or the source of blood.

Pima CD4 slightly underestimates the CD4 cell counts but the mean values were not significantly different from the reference method (FASCaliber flow cytometry) and the two machines were highly correlated (r = 0.96). Accuracy of Pima was similar to BD FACSCAliber. Thus, Pima CD4 can be used for CD4 enumeration particularly in resource-limited settings like rural Uganda.

Generally, CD4 counts enumerated by Pima CD4 for venous blood were higher than that for capillary blood but not significantly different (P=0.3142). Thus, rapid tests for CD4 counts can be performed on finger-prick blood samples instead of venous when dealing with small volumes of blood, in children or subjects with difficult to locate veins. This will potentially increase the accessibility for CD4 counting in resourcepoor settings, especially once rapid CD4 tests become widely available.

Further, within run precision demonstrated that the inherent imprecision of the Pima CD4 instrument is within the manufacturer's specifications and clinically acceptable limits. Also, between run precision demonstrated that the overall imprecision of Pima CD4 instrument and due to other external variants is within manufacturer's specifications and clinically acceptable limits.

The study revealed that Pima CD4 being a new technology, the operators are still committing errors: 24.3% errors with channel filling and reagent quality control being the most prominent where venous blood was used and 13.6% errors with image and reagent quality control for capillary blood the most common errors

6.2. Recommendations

Despite the few short comings, Pima CD4 may be suitable for health centers and remote areas with limited access to CD4 testing centers like rural Uganda. However, there is still need for proper and thorough training of the operators. Lastly, this study was done in a designated hospital HIV clinic environment. Thus, there is a need to evaluate the Pima CD4 in field conditions particularly in remote areas. Further feasibility studies should be conducted at health centre III and IV's and remote areas before Pima test can be rolled out at these levels of the health system.

REFERENCES

- Achuthanandam R, Quinn J, Capocasale RJ, Bugelski PJ, Hrebien L, Kam M. 2008. Sequential univariate gating approach to study the effects of erythropoietin in murine bone marrow. *Cytometry* **73**(8):702-714.
- Aina O, Dadik J, Charurat M, Amangaman P, Gurumdi S, Mang E, Guyit R, Lar N, Datong P, Daniyam C, Kanki P, and Abimiku A. 2005. Reference Values of CD4
 T Lymphocytes in Human Immunodeficiency Virus-Negative Adult Nigerians. *Clinical and Diagnostic Laboratory Immunology* 12(4): 525-530.
- Antonacci AC, Good RA, and Gupta S (1982). T-cell subpopulations following thermal injury. *Surgery, Gynecology and Obstetrics*; **155**(1); 1-8.
- Bagchi S, Kempf M, Westfall A, Maherya A, Wilig J, Saag M. 2007. Can routine clinical markers be used longitudinally to monitor antiretroviral therapy success in resource-limited settings? *Clinical Infectious Diseases* 44:135-137.
- Balakrishnan P, Solomon S, Mohanakrishnan J, Cecelia AJ, Kumarasamy N, Murugavel K, Venkatakrishnan B, Solomon S, Crowe S, Ganesh AK, Thyagarajan SP, Flanigan T, Mayer KH. 2006. A Reliable and Inexpensive EasyCD4 Assay for Monitoring HIV-Infected Individuals in Resource-Limited Settings. *Journal of Acquired Immune Deficiency Syndromes* 43 (1):23-26.
- Balter M.1997. How does HIV overcome the body's T-cell bodyguards? *Science* **278**: 1399-1400.
- Barbour JD. 2007. HIV-1/HSV-2 co-infected adults in early HIV-1 infection have elevated CD4+ T-Cell counts", *Public Library of Science since* ONE 2(10):e1080.
- Bartholomew C. 2001. Prices of CD4 assays and viral load tests must be reduced for developing countries. *British Medical Journal*. **323**:809-810.

- Beck JS, Potts RC, Kardjito T, and Grange JM (1985). T4 lymphopenia in patients with active pulmonary tuberculosis. *Clinical and Experimental Immunology*, **60**, 49-54.
- Bentley SA, Johnson A, Bishop CA. 1993. A parallel evaluation of four automated hematology analyzers. *American Journal Clinical Pathology* **100**:626-632.
- Bentwich Z. 2005. CD4 Measurements in Patients with HIV: Are They Feasible for Poor Settings? *PLoS Medicine Journal*. 2(7): e214.
- Bessinger R, Clark R, Kissinger P, Rice J, Coughlin S. 1998. Pregnancy is Not Associated with the Progression of HIV Disease in Women Attending an HIV Outpatient Program. *American Journal of Epidemiology* **147**(5):434-440.
- Biedermann K, Flepp M, Fierz W, Joller-Jemelka H, and Kleihues P: 1995. Pregnancy, immunosuppression and reactivation of latent toxo-plasmosis. *Journal of Perinatal Medicine* 1995, 23:191-203.
- Bisalinkumi E, Nawrocki P, Chao A, Bulterys M, Dushimimana A, Mugabo E, and Saah A 1992. T-cell subset changes during and after pregnancy in a cohort of HIV-1 sero(+) and sero(–) African mothers. VIIIth Int. Conf. AIDS, abstract. Number. PoA 2086. [Online.]

http://www.aegis.com/aidsline/1992/dec/M92C4967.html. Last accessed on March 21, 2010.

- Bland JM, and Altman DG. 1986. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **1**:307-310.
- Boedigheimer MJ, Ferbas J. 2008. Mixture modeling approach to flow cytometry data. *Cytometry* **73**(5):421-429.
- Bofill M, Janossy G, Lee CA, MacDonald-Burns D, Phillips AN, Sabin C, Timms A, Johnson MA, and Kernoff PB. 1992. Laboratory control values for CD4 and

CD8 T lymphocytes. Implications for HIV-1 diagnosis. *Clinical and Experimental Immunology* **88**:243-52.

- Bowen A, Palasanthiran P, Sohn AS. 2008. Global Challenges in the Development and Delivery of Paediatric Antiretrovirals. *Drug Discovery Today* **13**(11-12):530-535.
- Brando B, Barnett D, Janossy G, Mandy F, Autran B, Rothe G, Scarpati B, D'Avanzo G, D'Hautcourt JL, Lenkei R, Schmitz G, Kunkl A, Chianese R, Papa S, Gratama JW. 2000. Cytoflurimetric methods for assessing absolute numbers of cell subsets in blood. *Cytometry* 42:327-346.
- Brettle RP, Raab GM, Ross A, Fielding KL, Gore SM, and Bird AG. 1995. HIV infection in women: immunological markers and the influence of pregnancy. *AIDS* **9**(10):1177-1184.
- Burcham J, Marmor M, Dubin N, Tindall B, Cooper DA, Berry G, and Penny R.1991 CD4% is the best predictor of development of AIDS in a cohort of HIV-infected homosexual men. *AIDS* **5**:365-372.
- Burns DN, Landesman S, Minkoff H, Wright DJ, Waters D, Mitchell RM, Rubinstein A, Willoughby A, and Goedert JJ. 1998. The influence of pregnancy on human immunodeficiency virus type 1 infection: antepartum and postpartum changes in human immunodeficiency virus type 1 viral load. *American Journal Obstetrics and Gynecology* **178**(2):355-359.
- Burns DN, Nourjah P, Minkoff H, Korelitz J, Biggar RJ, Landesman S, Rubinstein A, Wright D, and Nugent RP. 1996. Changes in CD4+ and CD8+ cell levels during pregnancy and post partum in women seropositive and seronegative for human immunodeficiency virus-1. *American Journal Obstetrics and Gynecology*. 174(5):1461-1468.

- Castilla JA, Rueda R, Vargas L. 1989. Decreased levels of circulating CD4+ T lymphocytes during normal human pregnancy. *Journal Reproductive Immunology*; **15**:103-111.
- CDC 1981. Centers for Disease Control. Pneumocystis pneumonia Los Angeles. Morbidity and Mortality Weekly Report **30**:250-252.
- CDC 1989. Centers for Disease Control. Guidelines for prophylaxis against Pneumocystis carinni for persons infected with human immunodeficiency virus *Morbidity and Mortality Weekly Report* **38**:S4-S6.
- Chama CM, Morrupa JY, Abja UA, and Kayode A. 2009. Normal CD4 T-lymphocyte baseline in healthy HIV-negative pregnant women. *Journal of Obstetrics and Gynecology* **29**(8): 702-704.
- Chester D. 2008. Evaluating Assay Precision. *Clinical Biochemist Reviews*. 29(Supp. (i)): S23–S26.
- Clerici, Mario, Butto, Stefano, Lukwiya, Matthew, Saresella, Marina, Declich, Silvia, Trabattoni, Daria, Pastori, Claudia, Piconi, Stefania, Fracasso, Claudio, Fabiani, Massimo, Ferrante, Pasquale, Rizzardini, Giuliano, Lopalco, and Lucia, 2000.
 Immune activation in Africa is environmentally-driven and is associated with upregulation of CCR5. Italian–Ugandan AIDS Project. *AIDS* 14:2083–2092.
- CLSI 2004. Clinical and Laboratory Standards Institute. Evaluation of precision performance of quantitative measurement methods. *Wayne, PA, USA: CLSI; CLSI document EP05-A2.*
- Cracknell SE, Hinchliffe RF, Lilleyman JS. 1995. Lymphocyte subset counts in skin puncture and venous blood compared. *Journal of Clinical Pathology* **48**:1137-1138.

Crowe S, Mills J, McGrath MS. 1987. Quantitative immunocytofluorographic

analysis of CD4 surface antigen expression and HIV infection of human peripheral blood monocyte/macrophages. *AIDS Res Hum Retroviruses*. **3**(2):135–145.

- Crowe S, Turnbull S, and Oelrichs R. 2003. Monitoring of human immunodeficiency virus infection in resource-constrained countries. *Clinical Infectious Diseases* 37 (Suppl 1):S25-S35.
- Daae LNW, Halvorsen S, Mathisen PM, Mironska K. 1988. A comparison between haematological parameters in 'capillary' and venous blood from healthy adults. Scandinavian Journal of Clinical and Laboratory Investigation. 48:723-726.
- Danisman F, Van Benthem BH, Coutinho RA, and Prins M. 2001. The impact of pregnancy and menopause on CD4 counts in HIV-infected women. Conference on Retroviruses and Opportunistic Infections. Program Abstract 8th Conference Retroviruses Opportunistic Infections Conf Retrovir Oppor Infect 8th 2001 Chicago Ill. 2001 Feb 4-8; 8: 102 (abstract no. 205).
- Dayama A, Pandit S, Mudaliar R, Bharadwaj R, Shrotri AN, and Joshi S. 2003. A pilot study on CD4 and CD8 cell counts in healthy HIV seronegative pregnant women. *Indian Journal of Medical Research*. **117**:198-200.
- Deghaye N, Pawninski RA, and Desmond C. 2006. Financial and economic costs of scaling up the provision of HAART to HIV-infected health care workers in KwaZulu-Natal. South African Medical Journal. 96(2):140-143.
- Delmas MC, Jadand C, De Vincenzi I, Deveau C, Persoz A, Sobel A, Kazatchkine M, Brunet JB, Meyer L. 1997 Gender differences in CD4+ cell counts persist after HIV-1 infection. *AIDS* **11**:1071-1073.
- Denny TN, Gelman R, Bergeron M, Landay A, Lam L, Louzao R, Mandy FF, Schmitz J, Spira T, Wilkening C, Glencross DK; NIAID-DAIDS Immunology

Quality Assessment Program. 2008. A North American multilaboratory study of CD4 counts using flow cytometric panLeukogating (PLG): a NIAID-DAIDS Immunology Quality Assessment Program Study. *Cytometry B Clinical Cytometry* **74**(Suppl 1):S52-64.

- Department of Health and Human Services 2005. Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. April 7, 2005. *AIDSinfo* Web site (http://*AIDSinfo*.nih.gov).
- Deschamps MM, Fitzgerald DW, Pape JW, and Johnson WD, 2000. HIV infection in Haiti: natural history and disease progression. *AIDS*. **14**:2515–21.
- Diagbouga S, Chazallon C, Kazatchkine MD, van de Perre P, Inwoley A, M'Boup S, David MP, Tenin AT, Soudre R, Aboulker JP, Weiss L. 2003. Successful implementation of a low-cost method for enumerating CD4+ T lymphocytes in resource-limited settings: the ANRS 12-26 study. *AIDS*. 17:2201Y2208.
- Diagbouga S, Durand G, Sanou PT, Dahourou H, Ledru E. 1999. Evaluation of a quantitative determination of CD4 and CD8 molecules as an alternative to CD4+ and CD8+ T lymphocyte counts in Africans. *Tropical Medicine and International Health*. **4**:79-84.
- Didier JM, Katachkine MD, Demouchy C, Moat C, and Diagbouga S. 2001. Comparative assessment of five alternative methods for CD4+ T-lymphocyte enumeration for implementation in developing countries. *JAIDS Journal of Acquired Immune Deficiency Syndromes*. **26**:193-195.
- Ekpini and Gilks, 2005. Antiretroviral regimens for preventing HIV infection in infants. *Bulletin of the World Health Organization* **83**:7 Genebra July 2005

- Farzadegan H, Hoover DR, Astemborski J, Lyles CM, Margolick JB, Markham RB, Quinn TC, Vlahov D. 1998. Sex differences in HIV-1 viral load and progression to AIDS. *Lancet* 352:1510-1514.
- Giorgi JV, Cheng HL, Margolick JB, Bauer KD, Ferbas J, Waxdal M, Schmid I, Hultin LE, Jackson AL, Park L, and Taylor JMG. 1990. Quality control in the flow cytometric measurement of T-lymphocyte subsets: The Multicenter AIDS Cohort Study experience. *Clinical Immunology and Immunopathology* 55(2)173-186.
- Glencross D, Scott LE, Jani IV, Barnett D, Janossy G. 2002a. CD45-assisted PanLeucogating for accurate, cost-effective dual-platform CD4+ T-cell enumeration. *Cytometry*. **50**:69-77.
- Glencross DK, Coetzee L, Lawrie D, Stevens W, Osih R. 2010. Encouraging Pointof-Care PIMA CD4 Testing Performance in a Laboratory Setting: Johannesburg, South Africa. CROI. 16-19 February, 2010. San Francisco, California, USA. Poster abstract no. V-1003.
- Glencross DK, Stevens G, Scott LE, Mendelow BV, Stevens W. 2002b. The challenge of laboratory monitoring of HIV. South African Medical Journal. 92:248.
- Gosink JJ, Means GD, Rees WA, Su C, Rand HA. 2009. Bridging the divide between manual gating and bioinformatics with the bioconductor package flowFlowJo. *Advances in Bioinformatics* **2009**:1-8.
- Gratama JW, Kraan J, Keeney M, Granger V, and Barnett D. 2002. Reduction of variation in T-cell subset enumeration among 55 laboratories using single-platform, three or four-color flow cytometry based on CD4 and SSC-based

gating of lymphocytes. *Clinical Cytometry* **50**(2):92-101.

- Grinszte B, Smeaton L, De Gruttola V, Barnett R, Currier J, Swann E, Hakim J, Flanigan T, Kumarasamy J, and Campbell T 2008. Gender-associated differences in pre-ART plasma HIV-1 RNA in diverse areas of the world vary by CD4+ cell count. 15th CROI. February 2008. Boston, USA. Poster abstract 672.
- Grossman CJ. 1985. Interactions between the gonadal steroids and the immune system. *Science* **227**:257-60.
- Hegde HR, Woodman RC, Sankaran K. 1999. Nutrients as modulators of anergy in acquired immune deficiency syndrome. *Journal of Association of Physicians of India*; **47**(3): 318-25
- Hocke C, Morlat P, Chene G, Dequae L, and Dabis F, Group d'Epidémiologie
 Clinique du Sida en Aquitaine. 1995: Prospective cohort study of the effect of
 pregnancy on the progression of human immunodeficiency virus infection.
 Obstetrics and Gynecology 86:886-891.
- Hogan CM, and Hammer SM. 2001. Host determinants in HIV infection and disease.Part 1: cellular and humoral immune responses. *Annals of Internal Medicine* 134: 761-776.
- Hoover DR, Graham NM, Chen B, Taylor JM, Phair J, Zhou Y, Muñoz A. 1992. Effects of CD4+ cell count measurement variability on staging HIV-1 infection. *JAIDS Journal of Acquired Immune Deficiency Syndromes*. **5**:794-802.
- Huff B. 2001. Low cost diagnostics. *Gay Men's Health Crisis Treatment Issues*. **15**:1-4.
- Imade GE., Badung B., Pam S, Agbaji O, Egah D, Sagay AS, Sankalé JL, Kapiga S, Idoko J, Kanki P. 2005. Comparison of a New, Affordable Flow Cytometric Method and the Manual Magnetic Bead Technique for CD4 T-Lymphocyte

Counting in a Northern Nigerian Setting. *Clinical and Diagnostic Laboratory Immunology*. **12**(1): 224–227.

- Inverness Medical group, 2009. PimaTM CD4 Cartridge Guide. http://pimatest.com/fileadmin/Media/Downloads/Manuals/PI-PIMA-01-02.pdf. Accessed on December 02, 2009.
- ITPC. 2007. International Treatment Preparedness Coalition (ITPC). Missing the target #5: Improving AIDS drug access and advancing health care for all, December 2007. http://www.aidstreatmentaccess.org. Accessed May 10, 2010.
- Janossy G, and Jani I. 2000. Inexpensive CD4+ T lymphocyte counting by flow cytometry; relevance to developing countries. *International Conference on AIDS* **13**:28.
- Janossy G, Jani IV, Bradley NJ, Bikoue A, Pitfield T. 2002a. Affordable CD4+-T-cell counting by flow cytometry: CD45 gating for volumetric analysis. *Clinical and Diagnostic Laboratory Immunology* **9**:1085-1094.
- Janossy G, Jani IV, Kahan M, Barnett D, Mandy F. 2002b. Precise CD4 T-cell counting using red diode laser excitation: For richer, for poorer. *Cytometry B Clinical Cytometry*. **50**:78-85.
- Josefowicz S, Louzao R, Lam L, Ding T, Bergeron M, Henderson L, Mulder C, Tyagarajan K, Buchner L, Sinclair E, Denny T, Spira T, Mandy F, Landay A, Bredt B. 2005. 5-Site evaluation of the Guava EasyCD4 assay for the enumeration of human CD4+ T cells. Paper presented at: 12th Conference on Retroviruses and Opportunistic Infections. Boston, MA. Abstract 741.
- Kandathil AJ, Kannangai R, David S, Nithyanandam G, Solomon, S, Balakrishnan P,Abraham OC, Subramanian S, Rupali P, Verghese VP, Pulimood S, SridharanG. 2005. Comparison of Microcapillary Cytometry Technology and Flow

Cytometry for CD4+and CD8+ T-Cell Estimation Clinical and Diagnostic *Laboratory Immunology* **12**(8):1006–1009.

- Kannangai R, Prakash KJ, Ramalingam S, Abraham OC, Mathews KP. 2000. Peripheral CD4+/CD8+ T-lymphocyte counts estimated by an immunocapture method in the normal healthy south Indian adults and HIV seropositive individuals. *Journal of Clinical Virology*. 17:101-108.
- Kannangai R, Ramalingam S, Jesudason MV, Vijayakumar TS, Abraham OC. 2001.
 Correlation of CD4+ T-Cell counts estimated by an immunocapture technique (Capcellia) with viral loads in human immunodeficiency virus-seropositive individuals. *Clinical and Diagnostic Laboratory Immunology*. 8:1286-1288.
- Karcher H, Böhning D, Downing R, Mashate S, Harms G. 2006. Comparison of Two Alternative Methods forCD4+ T-Cell Determination (Coulter Manual CD4 Count and CyFlow) Against Standard Dual Platform Flow Cytometry in Uganda. *Cytometry Part B (Clinical Cytometry)* **70B**:163-169.
- Koonin LM, Ellerbrock TV, Atrash HK,Rogers MF, Smith JC, Hogue CJR, Harris MA, Chavkin W, Parker AL, and Halpin GJ. 1989. Pregnancy-associated deaths due to AIDS in the United States. *Journal of the American Medical Association* 261(10):1306-9.
- Kovacs JA, and Masur H. 2003. Prophylaxis against opportunistic infections in patients with human immunodeficiency virus infection. *The New England Journal of Medicine* **342**:1416-1429.
- Landay A, Ho JL, Hom D, Russell T, Zwerner R. 1993. A rapid manual method for CD4+ T-cell quantitation for use in developing countries. *AIDS*. **7**:1565-1568.
- Leroy V, Karon JM, Alioum A, Ekpini ER, Meda N, Greenberg AE, Msellati P, Hudgens M, Dabis F, and Wiktor SZ, <u>West Africa PMTCT Study Group</u>. 2002.

Twenty-four month efficacy of a maternal short-course zidovudine regimen to prevent mother-to-child transmission of HIV-1 in West Africa. *AIDS* **16**:631-641.

- Lindgren S, Anzén B, Bohlin AB, and Lidman K. 1991. HIV and childbearing: clinical outcome and aspects of mother to infant transmission. *AIDS* **5**(9):1111-1116.
- Linnet K, and Boyd JC. 2006. Selection and analytical evaluation of methods with statistical techniques. In: Burtis CA, Ashwood ER, Bruns DE, editors. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 4. *Elsevier Saunders; St Louis.* p. 357.
- Lo K, Brinkman RR, Gottardo R. 2008. Automated gating of flow cytometry data via robust model-based clustering. *Cytometry* **73**(4):321-332.
- Lutwama F, Serwadda R, Mayanja-Kizza H, Shihab HM, Ronald A, Kamya MR, Thomas D, Johnson E, Quinn TC, Moore RD, Spacek LA. 2008. Evaluation of Dynabeads and Cytospheres Compared With Flow Cytometry to Enumerate CD4+ T Cells in HIV-Infected Ugandans on Antiretroviral Therapy. JAIDS Journal of Acquired Immune Deficiency Syndromes. 48(3): 297-303.
- Lyamuya EF, Kagoma C, Mbena EC, Urassa WK, Pallangyo K. 1996. Evaluation of the FACScount, TRAx CD4 and Dynabeads methods for CD4 lymphocyte determination. *Journal of Immunological Methods*. **195**:103-112.
- MacLennan CA, van Oosterhout JJG, White SA, Drayson MT, Zijlstra EE, Molyneux ME. 2007. Finger-prick blood samples can be used interchangeably with venous samples for CD4 cell counting indicating their potential for use in CD4 rapid tests. *AIDS*. **21**(12):1643-1645.

- Maini MK, Gilson RJ, Chavda N,Gill S, Fakoya A, Ross EJ, Phillips AN and Weller IV. 1996. Reference ranges and sources of variability of CD4 counts in HIVseronegative women and men. *Genitourin Med* 72:27-31.
- Malone JL, Simms TE, Gray GC, Wagner KF, Burge JR, and Burke DS. 1990. Sources of variability in repeated T-helper lymphocyte counts from human immunodeficiency virus type-1 infected patients: total lymphocyte count fluctuations and diurnal cycle are important. *Journal of Acquired Immune Deficiency Syndromes* 3:144-51.
- Mayanja-Kizza H., Jones-Lopez E., Okwer, A., Wallis RS, Ellner JJ, Mugerwa RD,
 Whalen CC. 2005. Immunoadjuvant prednisolone therapy for HIV-associated
 tuberculosis: a phase 2 clinical trial in Uganda. *Journal of Infectious Diseases*.
 2005 191(6):856-65.
- McLachlan G, and Peel D. 1998. Robust cluster analysis via mixtures of multivariate t-distributions. In: Proceedings of the Joint IAPR International Workshops on Advances in Pattern Recognition, vol. 1451; January 1998; pp. 658–666. Lecture Notes in Computer Science.
- Mellors JW, Munoz A, Giorgi JV, Margolick JB, Tassoni CJ. 1997. Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Annals of Internal Medicine*. **126**:946-954.
- Messele T, Abdulkadir M, Fontanet AL, Petros B, Hamann D, Koot M, Roos MT, Schellekens PT, Miedema F, Rinke de Wit TF, 1999. Reduced naive and increased activated CD4 and CD8 cells in healthy adult Ethiopians compared with their Dutch counterparts. *Clinical and Experimental Immunology* **115**:443– 450.
- Miotti PG, Liomba G, Dallabetta GA, Hoover DR, Chiphangwi JD, and Saah AJ.

1992. T-lymphocyte subsets during and after pregnancy: analysis in human immunodeficiency virus type-1-infected and uninfected Malawian mothers. *Journal of Infectious Diseases* **165**(6):1116-19.

- Mocroft A, Jill MJ, Davidson W, Phillips AN 2000. Are there gender differences in starting protease inhibitors, HAART, and disease progression despite equal access to care? *Journal of acquired immune deficiency syndromes*. **24**(5):475-482
- Naumann U, Luta G and Wand MP, 2010. The curvHDR method for gating flow cytometry samples. *BioMed Central Bioinformatics*, **11**:44doi:10.1186/1471-2105-11-44
- Newell M-L, Rudin C, Dunn D, Peckham C. 1997. Immunological markers in HIVinfected pregnant women. *AIDS* **11**(15):1859-1865.
- Nicholson JK, Velleca WM, Jubert S, Green T, and Bryan L. 1994. Evaluation of alternative CD4 technologies for the enumeration for CD4 lymphocytes. *Journal of Immunological Methods* **28**:43-54.
- Nicholson JKA, Stein D, Mui T, Mack R, Hubbard M, Denny T. 1997. Evaluation of a Method for Counting Absolute Numbers of Cells with a Flow Cytometer. *Clinical and diagnostic laboratory immunology* **4**(3):309-313.
- Nightingale SD, Lawrence AJ, Wendell G, and Edison R 1992. CD4 count and pregnancy in HIV+ women. *International AIDS Conference* 8: B96 (abstract no. PoB 3056).
- Norris PJ, and Rosenberg ES. 2002. CD4 (+) T helper cells and the role they play in viral control. *Journal of Molecular Medicine* **80**: 397-405.
- O'Brien WA, Hartigan PM, Daar ES, Simberkoff MS, Hamilton JD. 1997. Changes in plasma HIV RNA levels and CD4+ lymphocyte counts predict both response to

antiretroviral therapy and therapeutic failure. VA Cooperative Study Group on AIDS. *Annals of Internal Medicine*. **126**:939-945.

- O'Brien WA, Hartigan PM, Martin D, Esinhart J, Hill A. 1996. Changes in plasma HIV-1 RNA and CD4+ lymphocyte counts and the risk of progression to AIDS. Veterans Affairs Cooperative Study Group on AIDS. *The New England Journal of Medicine*. **334**:426-431.
- Pattanapanyasat K, and Thakar MR. 2005. CD4+ T cell count as a tool to monitor HIV progression & anti-retroviral therapy. *Indian Journal of Medical Research* 121(4):539-549.
- Peter T, Badrichani A, Wu E, Freeman R, Ncube B, Ariki F, Daily J, Shimada Y, Murtagh M, 2008 Challenges in implementing CD4 testing in resource-limited settings. Cytometry Part B; 74B (Suppl. 1): S123–S130.
- Peto T. 1996. Surrogate markers in HIV disease. *Journal of Antimicrobial Chemotherapy* **37**(suppl. B): 161-170.
- Prins M, Robertson JR, Brettle RP, Aguado IH, Broers B, Boufassa F, Goldberg DJ, Zangerle R, Coutinho RA, van den Hoek A. 1999. Do gender differences in CD4 cell counts matter? *AIDS* 13 (17): 2361-2364.
- Pugach P, Kuhmann SE, Taylor J, Marozsan AJ, Snyder A, Ketas T, Wolinsky SM, Korber BT, Moore JP. 2004. The prolonged culture of human immunodeficiency virus type 1 in primary lymphocytes increases its sensitivity to neutralization by soluble CD4. *Virology* 21(1,):8-22.
- Pynea S, Hua X, Wang K, Rossina E, Linc T, Maiera LM, Baecher-Alland C, McLachlanb GJ, Tamayoa P, Haflera DA, De Jagera PL, Mesirova JP. 2009. Automated high-dimensional flow cytometric data analysis. Proceedings of the National Academy of Sciences of the United States of America.

- Quinn TC. 2003. World AIDS Day: Reflections on the pandemic. *The Hopkins HIV Report* **15**:12-14.
- Rachlis AR, Zarowny DP. 1998. Guidelines for antiretroviral therapy for HIV infection. Canadian HIV Trials Network Antiretroviral Working Group. *Canadian Medical Association Journal* 158:496-505.
- Rodriguez WR, Christodoulides N, Floriano PN, Graham S, Mohanty S, Dixon M,
 Hsiang M, Peter T Zavahir S, Thior I, Romanovicz, D, Bernard D, Goodey AP,
 Walker BD, McDevitt, JT. 2005. A microchip CD4 counting method for HIV
 monitoring in resource-poor settings. *PLoS Medicine*. 2:e182.
- Roederer M, and Hardy RR. 2001. Frequency difference gating: a multivariate method for identifying subsets that differ between samples. *Cytometry*.**45** (1):56-64.
- Saada M, Le Chenadec J, Berrebi A, Bongain A, Delfraissy JF, Mayaux MJ, Meyer L. 2000. Pregnancy and progression to AIDS: results of the French prospective cohorts. *AIDS* 14(15):2355-2360.
- SAS Institute Inc. 2008. SAS/STAT Software: Reference, Version 9.2, Cary, NC: SAS Institute Inc.
- Satoh C, Dan K, Yamashita T, Jo R, Tamura H, Ogata K. 2008. Flow cytometric parameters with little interexaminer variability for diagnosing low-grade myelodysplastic syndromes. *Leukemia Research*. 32(5):699-707.
- Scott GB, Fischl MA, Klimas N, Fletcher MA, Dickinson GM, Levine RS, Parks WP. 1985. Mothers of infants with the acquired immunodeficiency syndrome acquired immunodeficiency syndrome. *The Journal of the American Medical Association* 253(3):363-366.

Sherman GG, Galpin JS, Patel JM, Mendelow BV, and Glencross DK. 1999. CD4+ T

cell enumeration in HIV infection with limited resources. *Journal of Immunological Methods*. **222**:209-217.

- Smith CJ, Sabin CA, Youle MS, Kinloch-de Loes S, Lampe FC, Madge S, Cropley I, Johnson MA, and Phillips AN. 2004. Factors influencing increases in CD4 cell counts of HIV-positive persons receiving long-term highly active antiretroviral therapy. *Journal of Infectious Diseases*. **190**(10):1860-1868.
- Spacek, LA, Shihab HM, Lutwama F, Summerton J, Mayanja H, Ronald A, Margolick JB, Nilles TL, Quinn TC. 2006. Evaluation of a Low-Cost Method, the Guava EasyCD4 Assay, to Enumerate CD4-Positive Lymphocyte Counts in HIV-Infected Patients in the United States and Uganda *Journal of Acquired Immune Deficiency Syndromes* 41(5):607-610.
- Sridama V, Pacini F, Yang S, Moawad A, Reilly M, and DeGroot LJ. 1982. Decreased levels of helper T cells: a possible cause of immunodeficiency in pregnancy. *The New England Journal of Medicine*. **307**:352-356.
- Sterling T, Lyles C, Quinn TC, Astermborski J, Margolick J, Macalino G, Vlahov D. 1998. Gender-specific differences in HIV-1 RNA level in a longitudinal study of HIV-1 seroconverters. XIIth International Conference on AIDS, Geneva, June 1998. Abstract 13379.
- Sugar CA, James GM. 2003. Finding the number of clusters in a dataset: an information-theoretic approach. *Journal of the American Statistical Association* 98(463):750-763.
- Taylor JM, Fahey JL, Detels R, and Giorgi JV. 1989. CD4 percentage, CD4 number and CD4:CD8 ratio in HIV infection. Which to choose and how to use. *Journal* of Acquired Immune Deficiency Syndromes **2**:114-124.

Teav S, Lynen L, Vereecken C, De Munter P, Srey C, Kestens . 2004. Alternative

CD4 counting using Cyflow in Cambodia: precision and comparison with Facscount. *International Conference AIDS*. 2004 Jul 11-16; **15**: abstract no. MoPeB3089.

- Temmerman M, Nagelkerke N, Bwayo J, Chomba EN, Ndinya-Achola J, and Piot. P. 1995. HIV-1 and immunological changes during pregnancy: a comparison between HIV-1 seropositive and HIV-1 seronegative women in Nairobi, Kenya. *AIDS* 9:1057.
- UNAIDS, 2008. Report on the global AIDS epidemic
- UNAIDS/WHO 2003. UNAIDS/WHO AIDS Epidemic Update Part II.; http://data.unaids.org/pub/EPISlides/2007/2007_epiupdate_en.pdf Accessed: [12 October 2009]
- UNAIDS/WHO 2007. AIDS epidemic update. Special Report on HIV/AIDS, December 2007. WHO Geneva Switzerland: 2007. [Accessed 12 October 2009]
- USAID, 2006. USAID | DELIVER PROJECT, Task Order 1. 2008. Building a Standard Laboratory Equipment List. Arlington, Va.: USAID | DELIVER PROJECT, Task Order 1.
- Van Blerk M, Bernier M, Bossuyt X, Chatelain B, D'Hautcourt JL, Demanet C, Kestens L, Van Bockstaele D, Crucitti T, Libeer JC. 2003. National external quality assessment scheme for lymphocyte immunophenotyping in Belgium. *Clinical Chemistry and Laboratory Medicine*. **41**(3):323-330.
- Villas BH. 1998. Flow cytometry: An overview. The Journal of Cell Biology 5:56-61.
- Weinberg ED. 1984. Pregnancy-associated depression of cell-mediated immunity. *Reviews of Infectious Diseases* 6:814-831.
- Weisser M, Rudin C, Battegay M, Pfluger D, Kully C, Egger M. 1998. Does pregnancy influence the course of HIV infection? Evidence from two large Swiss

cohort studies. Journal Acquired Immune Deficiency Syndromes 17(5):404-410.

- WHO 2003. World Health Organisation. Treating 3 million by 2005: Making it happen. Geneva.
- WHO 2004a. Joint United Nations Programme on HIV/AIDS, World Health Organization. AIDS epidemic update: 2004. 2004 December.
- WHO 2004b. World Health Organization. World Health Organization (2004) 3 by 5 progress report: December 2003 through June 2004; 2004.
- WHO 2004c. World Health Organization. Antiretroviral drugs for treating pregnant women and preventing HIV infection in infants. Guidelines on care, treatment and support for women living with HIV/AIDS and their children in resourceconstrained settings. <u>http://www.who.int/hiv/pub/mtct/en/arvdrugswomen</u> guidelinesfinal.pdf. Last accessed on May 12, 2010.
- WHO 2006. Peer Review of Regional Paediatric Antiretroviral Treatment Guidelines. A Report. New Delhi, India, 4-5 May 2006 WHO Project: ICP HIV 500. http://203.90.70.117/PDS_DOCS/B0346.pdf. Last accessed on May 02, 2010.
- WHO 2007. Laboratory Guidelines for enumerating CD4 T Lymphocytes in the context of HIV/AIDS. Regional Office for South-East Asia. New Delhi, June 2Appendix 1:
- WIC 2009. Walta Information Center. Speedy CD4 count results in HIV positive patients crucial?http://www.waltainfo.com/index2.php?option=com_content &dopdf =1&id=16485. Last accessed April 20, 2010.
- Williams RC, Koster FT, Kilpatrick KA (November, 1983). Alterations in lymphocyte cell surface markers in various human infections. *American Journal* of Medicine: **75**; 807-816.

Yang Z-W, Yang S-H, Chen L, Qu J, Zhu J, Tang Z. 2001. Comparison of blood

counts in venous, finger-prick and arterial blood and their measurement variation. *Clinical and Laboratory Haematology*. **23**:155-159.

- Yu LM., Easterbrook PJ., and Marshall T. 1997. Relationship between CD4 count and CD4% in HIV-infected people. *International Journal of Epidemiology* 26(6):1367-1372.
- Zijenah LS, Kadzirange G, Madzime 2006. Affordable flow cytometry for enumeration of absolute CD4⁺ T-lymphocytes. *Journal of Translational Medicine* **4:**33.

Appendix I: Gating of anticipated cell population distribution:

Adopted from Thakar et al., (2006)



- CD3+ cells (in red) are gated using the size (FSC: X axis) and the CD3-PECy5 staining (PM2: Y axis) using the threshold setting markers.
- CD3+CD4+ T cells are gated in CD4 analysis gate using two-color fluorescence CD4- PE (PM1: X axis) and CD3- PECy5 (PM2: Y axis) using the threshold setting markers.

Appendix II: Study Schema



Appendix III: RESEARCH SUBJECT INFORMATION SHEET

Purpose

We invite you to take part in a research study because you have confirmed HIV infection and have presented to Mulago Hospital. Part of monitoring HIV infection is the measurement of T-helper cell count (CD4), an indicator of your immune status. A new method has been developed to determine T-helper cell count within approximately 20 minutes in one drop of blood from your finger and intend to compare the results of this new method with an already established laboratory test.

What should you know about your participation in this research study?

Your participation in this study is voluntary and will take about 20 minutes of your time. You have the right to refuse to take part in this study, or you may agree to take part and change your mind later. Whatever you decide, it will not affect your regular care.

What procedures will be done to you as part of this study?

We will collect about one drop of blood from two separate fingersticks (about two drops of blood total; this may be repeated if the second finger prick was not successful)

We will also collect about one tablespoon (8mL) of blood from your vein

The blood samples will be tested with the new method. Your age, gender, and some information about your medical history, diagnosis and whether you receive ART will

also be collected for this study. However, no information that can directly identify you will be available to the researchers.

Your blood will also be tested for T-helper cell count with the routine method at Mulago Hospital and the result compared to the new method.

The new method is not yet approved for medical use and your physician will not use the results to evaluate your condition. You will still receive the standard care that you would normally receive while in the clinic.

What are the possible benefits?

There are no direct health benefits to you for taking part in this study.

What are the possible risks and discomforts?

Blood will be drawn from your arm and by fingerprick. Risks associated with drawing blood include pain, bruising or swelling at the site of the blood draw; Infection at the site of the blood draw is also possible, but very unlikely.

For more information about risks and side effects, ask the investigator or nurse.

What information will be kept confidential?

Information collected for this study will be reported to the study investigator. The reported information will be coded by a number to protect your identity.

RESEARCH SUBJECT INFORMED CONSENT FORM

Investigator:

"I have read, or it was read to me, the information sheet concerning this study and I
understand what will be required of me if I take part in the study"
"I am aware of the possible risk and benefits of this study"
"I know that being in this study is voluntary"
"I understand that at anytime I may withdraw from this study without giving a reason
and without affecting my normal care"
"My questions concerning this study have been answered by"
"I agree to take part in this study"
SIGNATUREDATE
NAME (Please Print)
Signature of Person Obtaining the Informed Consent:
SIGNATUREDATE
NAME (Please Print)
Appendix IV: Facscalibur daily operations SOP
1. Samples Preparation Procedure (Lyse No-Wash Staining)
1.1. Label two 12 x 75 mm Trucount tubes with CD-Chex Plus QC Normal
& Low Properly label 12 x 75 mm Trucount tube with specimen ID

1.2. Add 20 µl of the Antibody (Ab) reagent (Multitest

CD3/CD8/CD45/CD4) to the appropriate tubes; pipette the Ab reagent just above the steel retainer.

- 1.3. Gently invert the specimen tube to mix and then carefully remove the stopper (make sure you follow Universal Biohazards Precautions)
- 1.4. Add 50ul blood to each tube just above the retainer using a pipettor. The reverse pipetting method should be used to ensure the correct volume is added.
- 1.5. Cap the Trucount tube and vortex gently to mix
- 1.6. **Incubate** at RT (20 to 25°C) **in the dark for 15 minutes** (utilize a timer to ensure time accuracy).
- 1.7. Adjust the 1000 ul pipette to 450 ul. Add 450 ul of DilutedFACSLyse (1:10) to each tube and vortex after each addition.
- 1.8. **Incubate at RT for 15 min** in the dark to lyse the red blood cells.
- 1.9. Analyze on FACSCalibur flow cytometer immediately. Samples can be stored in the dark at room temperature (RT) until ready to analyze, however, they should be run on the flow cytometer within 8 hours after staining (can be kept up to 48hr @2-8C). Vortex each tube gently before placing on the FACSCalibur.

93

2. QUALITY CONTROL (QC TESTING)

- 2.1. As indicated in point #8.1 each set of daily specimens is run with stabilized blood product with two levels of control i.e. CD-Chex Plus QC Normal & Low
- 2.2. The QC samples are run and prepared just like patient samples except for:
 - 2.2.1. The QC samples are placed on the carousel in positions #1 & 2
 - 2.2.2. For QC samples, we must select the QC testing file from the MultiTest software.
- 2.3. Each QC lot comes with a package insert listing the ranges of values for CD3, CD4, and CD8. The QC results obtained are compared to those listed on the insert and they must be within the documented range.
- 2.4. If analyzed QC's values are within acceptable range, accept results (instrument will save QC results in the FacsComp software) print results and save in the FacsCalibur QC log, then document as "OK" on the Core Lab daily QC review log, and proceed with running & accepting patient samples results.
- 2.5. If the analyzed QC's are not both within the specified range then the CD-Chex Plus QC standard controls failed and the following steps are to be performed in order to proceed with the patient sample analysis.
 - 2.5.1. Do not analyze the patient samples.
 - 2.5.2. Repeat a freshly prepared CD-Chex Plus QC

Note: If QC OK this time, we need to re-prepare all patient samples done on the same batch (if any). To ensure the sample preparation process is OK as well.

2.5.3. If the freshly prepared D-Chex Plus QC fails again then do not analyze the patient samples, proceed to step 9.5.4 below.

2.5.4. Inform the Lab Supervisor or team lead, and a different operator stains a fresh D-Chex Plus QC sample and all patient samples. Analyze the QC and if it passes then proceed with analyzing the re-stained patient samples
Appendix V: QUICK REFERENCE GUIDE

1. Turn ON the Pima Analyzer and wait until this message appears on the screen. Press <OK>

and Pima is ready for a new analysis

2. Remove Pima CD4 Test Cartridge from its protective pouch.

3 Select a site for fingerstick.

- The patient should be sitting or lying down.
- Select a collection site (3rd or 4th finger are preferable as shown in the illustration).
- Select an area off the centre of the finger pad (shaded area).

4 Warm the fingers if necessary.

• Have the patient hold their hand downwards to increase blood flow to the fingers.

5 Wipe the tip of the selected finger with alcohol and allow the alcohol to air dry

- 6 Perform fingerstick
- Use the sterile lancets provided by the

Sponsor to make a skin puncture just off the centre of the finger

pad.

- It is important to press lancet firmly onto the finger and maintain contact while ejecting the lancet.
- 7 Collect specimen
- If necessary, massage gently from the hand to near the puncture site.







- Wipe away the first drop of blood with a sterile gauze pad.
- Position end-to-end capillary of the cartridge directly in contact with the blood drop and allow the capillary to fill.

8 Observe the control window to ensure sufficient loading.

• Remove cartridge from finger when area in the control window is filled with blood.



10 Remove protective label from the cartridge.









11 completely insert cartridge into the Pima Analyzer in the direction indicated by the arrow on the cartridge label.

- 12 Enter Operator and Sample ID. Expect approximately 20 minutes for completion of the analysis.
- 13 Remove cartridge when prompted by the analyzer.
 - Discard used cartridge as biohazardous waste.
- 14 Test result is displayed on the screen.
- 15 Print Pima Test Report from current analysis and attach to the Source Document.

Appendix VI: QUICK REFERENCE GUIDE SAMPLE COLLECTOR

1. Remove one Sample Collector from the protective pouch

2 Hold the wide end of the Sample Collector between thumb and index finger so that the capillary points upwards.

3. Squeeze the wide end of the Sample Collector between thumb and index finger (like a clothes pin). Align the Sample Collector with the top end of the cartridge.

4. Place the Sample Collector so that its capillary slides over the capillary of the cartridge and push it downwards until it fits tight against the orange holding strap of the cartridge cap.







5. If done correctly the Sample Collector slides easily onto the two black pillars of the cartridge base. If you feel too strong resistance, squeeze harder on the wide end Sample Collector and check the alignment. Release the Sample Collector. It is now ready to be filled with blood.

6. Apply sample onto the sample collector until the collector is filled with blood (approx. 30uL).

7. Stop filling when collector is full.

8. Observe the control window to ensure sufficient loading. Hold the cartridge upwards, in a 45 degree angle. Do not remove the sample collector until the control window is filled with blood.









9. Squeeze the wide end of the Sample Collector between thumb and index finger and remove Sample Collector from the cartridge in one continuous upwards motion. Dispose as biohazardous waste.



10. Check that the small capillary is still in place.

11. Completely close the cartridge cap and insert the cartridge into the Pima Analyser.



Appendix VII: Ethical Approval



Uganda National Council for Science and Technology

(Established by Act of Parliament of the Republic of Uganda)

Our Ref: HS 630

11/09/2009

Dr. Yuka Manabe Principal Investigator Infectious Diseases Institute P.O Box 22418 Kampala.

Dear Dr. Yuka Manabe,

RE: APPROVAL TO CARRY OUT A RESEARCH AT INFECTIOUS DISEASES INSTITUTE-MAKERERE UNIVERSITY COLLEGE OF HEALTH SCIENCES.

We refer to your application to Uganda National Council of Science and Technology (UNCST) dated 2nd August 2009 requesting for permission to carry out an approved research project entitled Clinical Evaluation of the Accuracy of the CD4+ T-cell counts using PIMATM CD4 as compared to BD Facscaliber Flow Cytometry.

The UNCST, on 7th September 2009 approved your application to conduct the above research at Infectious Disease Institute between September and December 2009, on 250 HIV infected individuals.

The approval is subject to the terms and conditions of the Infectious Diseases Institute (IDI) clinic cohort committee. We also request that you submit to UNCST a report of the study findings.

Yours sincerely,

Leah Nawegulo for: Executive Secretary UGANDA NATIONAL COUNCIL FOR SCIENCE AND TECHNOLOGY

- c.c The Secretary, Office of the President
- c.c. Director, Mulago Hospital
- c.c. Chairperson, IDI Clinic Cohort Committee

Received at MUJHU

Date: 14 SEPT 2009

Initials: CN

Page 1 of 2

LOCATION/CORRESPONDENCE

Plot 6 Kimera Road, Ntinda P. O. Box 6884 KAMPALA, UGANDA COMMUNICATION

TEL: (256) 414 705500 FAX: (256) 414-234579 EMAIL: info@uncst.go.ug WEBSITE: http://www.uncst.go.ug
 TELEPHONE
 : Direct
 256-41-554008

 : Fax No
 256-41-5

 E-mail:
 d-mh@uga.healthnet.org



MULAGO HOSPITAL P. O. BOX 7051 KAMPALA, UGANDA.

IN ANY CORRESPONDENCE ON THIS SUBJECT PLEASE QUOTE...... THE REPUBLIC OF UGANDA

3rd September 2009

Mr. Kafufu Fred Bosco

Dear Mr. Kafufu Fred Bosco

RE: YOUR RESEARCH PROPOSAL ENTITLED: Evaluation Of The Accuracy Of The CD4+ T-Cell Counts Using Pima[™] CD4 As Compared To BD Facscaliber Flow Cytometry

This is to certify that your Research proposal by the above title was received, reviewed and passed by the Mulago Hospital Research and Ethics Committee.

You may now go ahead and conduct your research as outlined in your proposal and under the guidance of your stipulated supervisors. At completion of your study, you are required to provide us with a copy of your research report.

MINISTRY OF HEALTH Sincerely MULAGO HOSPITAL P. O. BOX PASI. KAMPALA

Prof. Seggane Musisi Chairman Mulago Research and Ethics Committee.

- c.c. Ms. Jesca Lukanga Nakavuma (PhD) Senior Lecturer, Microbiologist & Head, Dept. of Veterinary Parasitology & Microbiology.
- c.c. Dr. Othieno Emmanuel Supervisor
- c.c. Dr. Yuka Manabe Infectious Diseases Institute