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Comparative Study of CD4 Analytical Methods

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Abstract

Human immunodeficiency virus (HIV) infection is increasing at an alarming rate worldwide, and the burden is heaviest in sub-Saharan Africa. Obtaining accurate, reliable, and affordable CD4 T-lymphocyte counts is essential in determining disease stage and progression. It is also crucial in monitoring ARV therapy determining when to change antiretroviral (ARV) due therapy resistance or therapy failure since option B plus or test and start therapy is practiced now. Two CD4 T-lymphocyte counting measuring techniques compared: flow cytometry (Cyflow) and magnetic beads (Dynabead). This study was conducted at 1 Brigade Medical Centre Sokoto between May and August 2015. Blood samples (7 mls) were collected in EDTA Vacutainer tube from each of 200 adults consisting of; HIV-positive patients(15-60years) in order to obtain a wide range (low, medium, and high) of CD4 T-lymphocyte counts. All blood samples were analyzed in duplicate for CD4 T lymphocytes within 6 hours of collection. The results from both methods showed good correlation in the CD4 lymphocyte count of the 200 adult blood samples counted ($p = 0.071$, $r = 0.99$). The Cyflow technique is more precise and cost-effective than the Dynabeads method since as many as 100 samples can be measured per day. Although flow cytometry is the "gold standard" for accurate and automated measurement of CD4 T

lymphocytes, the technique is expensive and requires sophisticated equipment as well as trained personnel to perform it. On the other hand, manual non-flow cytometric techniques such as the Dynabeads and Coulter methods, which produce results comparable to those of flow cytometry, have their own limitations. They are labor-intensive, and only a few (10 to 15) CD4 T-lymphocyte counts can be performed by an analyst per day.

Keywords: Comparative Study, CD4, Analytical, Methods

Introduction

The HIV/AIDS pandemic has severely affected health development and eroded improvements in life expectancy, particularly in countries with the highest prevalence of infection. Since the first cases of Acquired Immunodeficiency Syndrome (AIDS) were reported in 1981, infection with human immunodeficiency virus (HIV) has grown to pandemic proportions, resulting in more than 65 million infections and 25 million deaths. At the end of 2006, an estimated 39.5 million people were living with HIV, with Sub-Saharan Africa carrying the highest burden (WHO, 2007). In 2006, an

estimated 4.3 million people became newly infected with HIV. More than 95% of these new infections are in low and middle-income countries. Each day 11,000 persons become newly infected with the virus; of these, half are women and 40% are young people (15-24 years old). Of the estimated 37 million adults living with HIV worldwide, nearly 18 million are women (WHO 2007). At the end of 2006, there were an estimated 2.3 million children living with HIV. The increasing number of child deaths due to AIDS threatens to reverse many of the recent gains of child survival programmes. Moreover, the socio-economic impact of HIV/AIDS on children is profound. As their parents fall sick and die of AIDS, children undergo a long trail of painful experiences such as: economic hardship, withdrawal from school; lack of love, attention and affection; psychological distress, stigma, discrimination and isolation, and malnutrition and illness. Cumulatively, there are a total of 16 million children worldwide who have become orphans because their parents died of AIDS (WHO, 2007).

Prevalence of HIV/AIDS in Nigeria

Nigeria is the most populous nation in Africa with an estimated population of over 140 million people. Government reports claim that over 300,000 Nigerians die yearly of complications arising from AIDS. Over 1.5 million children are said to be orphaned annually and an estimated 8 million are expected to be infected by 2012. Official figures put the steady rise in HIV prevalence rate from 1.8% in 1988 to 5.8% in 2001, 5.0% in 2003 and 4.4% in 2005.

The 2005 survey released in 2006 estimated that there were 4,000,000 adults living with HIV/AIDS in Nigeria, and 57% of these are women. From the figures, there is significant variation in prevalence between states and between population groups. The epidemic is said to be fuelled in the country largely by poverty, lack of awareness, dense commercial sex networks, early age of sexual debut, poor gender empowerment, with religion and culture obstructing open debate about sexuality. These challenges have impeded the progress of Nigeria's intervention effort, is corruption.

CD4 T lymphocytes and HIV

HIV has particular tropism for CD4 cells and destruction of CD4+ cells results in many of the deleterious effects of HIV. Within hours of exposure to HIV, CD4 T lymphocytes are found to be infected showing active viral replication. The infected CD4 cells release virions by budding through the cell membrane or by lysis of the infected cells. The released virus particles then infect uninfected CD4 T lymphocytes. CD4 T lymphocytes also serve as important reservoirs of HIV: a small proportion of these cells carry HIV provirus integrated in the host DNA without active virus multiplication (WHO, 2007).

During the primary HIV infection, the number of CD4 T lymphocytes in the bloodstream decreases by 20% to 40%. HIV brings about the lysis of HIV, infected cells as well as bystander uninfected cells using various mechanisms such as lysis of the cells infected with HIV. Billions of CD4 T lymphocytes may be destroyed every day, eventually overwhelming the immune system's regenerative capacity. In acute HIV-1 infection, in addition to the decline in CD4 T lymphocytes counts, qualitative impairments of CD4 T lymphocytes function are detected (WHO, 2007). The impairment of HIV-1-specific CD4 T lymphocytes function occurs very early in acute infection. Following acute primary HIV infection, one may remain free of HIV-related illnesses, often for years, despite ongoing replication of HIV in the lymphoid organs and relentless destruction of the immune system. However, during the period, the immune system remains sufficiently competent to provide immune surveillance and to prevent most infections. Although the decrease in the total number of T lymphocytes marks the decrease in immune competence, sometimes the quantitative loss of CD4 T lymphocytes may not be matched by the qualitative functions. A number of assays such as cytokine induction, antigen induced proliferation, measurement of activation markers etc can assess the functions of lymphocytes. However, the total CD4 T lymphocytes number still remains the most robust marker of immune competence (WHO, 2007).



The progressive loss of CD4 T lymphocytes eventually results in the loss of an ability to mount desirable immune response to any pathogen and vulnerability to opportunistic pathogens characteristic of AIDS. The estimation of peripheral CD4 T lymphocytes counts is relied upon for taking a decision on initiation of ART (WHO, 2007).

The estimation of peripheral CD4 T lymphocytes counts has also been used as a tool for monitoring disease progression and the effectiveness of antiretroviral treatment (ART). The changes in the CD4 T lymphocytes counts are important indicators of the response to ART. HIV plasma virus load is a sensitive indicator of the progression of HIV disease. However, due to the relatively high cost of virus load estimation, the CD4 T lymphocytes count remains the most important key indicator for initiation and monitoring of ART and a measure of the effectiveness of the treatment in clinical trial evaluations (WHO, 2007). Partec CyFlow volumetric system: CyFlow is another desktop single-platform technology made by Partec GmbH 32. It is a volumetric software controlled absolute count system equipped with either a single 532 nm green solid-state laser used for one fluorescence parameter or two lasers with a mercury arc lamp applicable for 2 or 3-colour analyses. Data acquisition and analysis are performed in real time with FlowMax software. The system showed good correlation with the CD4 counts obtained by conventional flow cytometry. However, experienced technician is required for accurate measurement. Although flow cytometry is the accepted standard method for the determination of absolute counts of CD4+ and CD8+ T-cells, the methodology involved the use of flow cytometer and haematology analyzer, which is expensive both in terms of initial investment and maintenance as well as requiring highly, trained personnel. Thus, it is unsuitable for routine use in most laboratories with limited facilities such as district hospitals. An ideal CD4 testing in a resource-poor setting would be an assay which is simple, uses inexpensive instrument and minimum training period that would reliably identify CD4+ T-cells without sacrificing much accuracy and precision. There are several alternative non-flow cytometric technologies available for determination

of absolute CD4+ and CD8+ T-cells. These technologies are cost-effective and thus might be suitable in the local situation in the resource-poor settings. Currently available manual assays rely on microscopes (optical or fluorescence). Additionally, they require the use of other small equipment such as centrifuges and magnets. These tests are designed to operate with low sample throughput in resource-limited laboratories but their running costs are not always lower when compared to the automated methods.

Dynabeads T4-T8 quantitative system: This non-flow cytometric technology is the product of Dynal. The system is based on the use of immunomagnetic cell isolation method which uses Dynabeads magnetic particles coated with antibody to CD4 and CD8 antigens to capture and isolate CD4+ and CD8+ T cells from the whole blood. In this system, the whole blood diluted with buffer solution is depleted of monocyte using monocyte depletion magnetic bead reagent coated with anti-CD14 antibody. After incubation on a Dynal rotator, magnetic separation of monocytes is done using a magnetic particle concentrator. Aliquot of the supernatant from the monocyte-depleted blood is diluted and mixed with magnetic beads coated with anti-CD4 monoclonal antibody. After incubation, the beads are separated from the CD4+ T-cells. The isolated CD4+ T-cells are then stained with gentian violet and trypan blue. The isolated stained cells are lysed and the nuclei are identified and counted manually by light microscopy or in an automated cell counter. The method has been validated in resource poor settings in Africa. The method showed significant correlation with that obtained by flow cytometry and found to be cost effective with US\$ 3/test.

However, these methods are manual and labour intensive. To scale these up to match-expanding access to ART may prove challenge. The automation by addition of image analysis software would increase the potential of its use in ART monitoring. The system could be cheaper than other alternatives and will be useful in small settings if it is backed up with flow cytometry for quality assurance.

Quality assurance in CD4 testing

Since the use of CD4+ T-cell measurement have critical implications for the effective management of individuals at risk or infected with HIV or progressing into AIDS, it is crucial that external quality control is put in place to ensure that the test results from individual laboratories are equivalent.

The aim of quality assurance is to ensure that doctors and patients are getting the same information from the laboratory tests, no matter which laboratory they use or the methods used there.

For a good quality control, the diurnal variation in the CD4 counts should be considered as an important factor. To avoid such variation, it is recommended that the sample should be collected at a specified time from an individual. Another point that has to be taken into account is the storage of sample before assessment. This period varies with the methodology used and location of the testing laboratory.

Several sets of guidelines addressing quality control of flow cytometric CD4+ testing have been developed. Though there is at present no external quality control on non-flow cytometric CD4 testing, it is important that accurate daily within laboratory (internal quality control) and proficiency test or external quality assurance programmes (EQA) be employed to ensure the reliability of CD4 data. Satisfactory performance in CD4 testing External Quality Assurance is recommended for HIV research and clinical trial programmes in many parts of the world most especially in developing country like Nigeria. For the most parts, internal control samples from the manufacturer are used to monitor both sample processing and instrument performance however, internal quality control practice is not without merit, it does not provide full-process quality control. There are very few laboratories, particularly in the developing world participating in international EQA programmes such as United Kingdom National External Quality A technology that is being developed and refined at present is a fixative that allows blood samples to be stored and transported over 5 days (or even slightly longer)

without loss of accuracy in test results. The high cost of these programmes has been a major burden, making their implementation in resource-poor countries difficult. However, there are free EQA programmes such as Quality Assessment and Standardization for Immunological Measures Relevant to HIV/AIDS (QASI), in spite of their irregular schedules; they are useful and cost saving.

Statement of Problem

HIV/AIDS patients are on the increase all over the globe most importantly in Nigeria. The use of highly active anti-retroviral (HAART) in the management of HIV/AIDS and its effectiveness can only be evaluated by estimation of the CD4 T lymphocyte to monitor the progress or effectiveness of the HAART. The method of CD4 estimation and the quality of the results has become important due to the dwindling support and poor setting in Nigeria.

Aim

The aim of this study was to assess the accuracy of different CD4 T lymphocyte analytical methods used in the monitoring of HIV/AIDS patients therapy.

Objectives of The Study

1. To evaluate the plasma CD4 T Lymphocyte of HIV/AIDS patients and the control group
2. To compare different CD4 T lymphocyte analytical methods used in the monitoring of HIV/AIDS patients and control.

Materials and Methods

Description of Study Area

Sokoto is one of the seven states that form the North West geopolitical zone of Nigeria. It is bordered to the North by the Republic of Niger, Zamfara State to the East, Kebbi State to the South and West. It is situated in the savannah on the temperature of 44 degree Celsius annually. The city of Sokoto is its capital. Sokoto State traces its origin to the Sokoto Caliphate founded in 1809 by Shehu Usman



Danfodio, the leader of the jihadists who overthrew the Hausa State of Gobir, Kano, Katsina and Kanem-Bornu. The empire fell after the British conquest of 1903 and the death of Attahiru, the Sultan of Sokoto, and became part of the Northern Region in the three-region structure of 1954. In 1967, Nigeria, the military administration of General Yakubu Gowon merged Sokoto and Niger provinces to form the North-Western State. In 1976, North Western State was split into Sokoto and Niger states by the military administration of General Murtala Muhammed.

Sokoto State covers an area of 28,232.37 square kilometers. The state is located between latitudes 40 to 60 North and longitudes 110 to 130 East has a population of 3,702,676 (2006 census figures). It accounts for 2.3 percent of Nigeria's total population. Prior to the establishment of Sokoto as a Ribat (military camp or frontier) in 1809, the area that is modern-day Sokoto state was home to Hausa state with large populations. These states eventually fell under the control of Usman Danfodio and the Fulani jihadists and became part of the Sokoto Caliphate. In 1817 when Usman died, his son Muhammed Bello succeeded him as the Sultan of Sokoto. Usman's brother Abdullahi was given the western divisions of the caliphate to run. However, supreme authority rested with Bello. At the height of its power, the Sokoto Caliphate extended as far as Ilorin (in modern-day Kwara State). The Hausa are the largest ethnic group in Sokoto State while the Fulani are its second largest. Minority include the Zabarmawa, Tuareg and the Dakarkari. The majority of the population is Sunni Muslim. There is a small Shia minority. There are twenty-three local government areas (LGAs) in Sokoto. Each has a chairman as its administrative head. The Islamic community in Nigeria considers the person of the Sultan as 'First among Equals'. He is both the political head of the Fulani as well as the supreme spiritual head of the roughly 70 million Muslims in Nigeria. Currently occupying the sit is Sultan Muhammadu Sa'ad Abubakar III, the twentieth Sultan of Sokoto. Agriculture is the mainstay of Sokoto's economy.

The riverine floodplains produce cash crops, including peanuts (groundnuts), cotton and rice.

sorghum, millet, cowpeas and cassava are grown in the upland areas. Much of the land in the state is used for grazing cattle. Cattle hides, goatskin, sheepskins and finished leather products are significant exports, as are cattle, goats and fowl. The state possesses limestone and kaolin deposits and Sokoto City. The State capital, is home to a cement factory, tanneries and a modern abattoir. Festivals include; Kalankuwa, Halbi, Sharo, AikinGawa, Shan Gumba-Pap drinking and Remo Fishing Festival. The stress associated with agriculture could increase the incidence of hypertension. The trend of cigarette smoking is very high among adult in Sokoto metropolis (Nigeria socio-economic indicator, 2012).

Ethical Consideration

Approval for the study was obtained from 1 Brigade Medical Centre Advisory Committee. Informed consent of the patients was also obtained. Patient's anonymity was maintained; the data generated was treated with strict confidentiality, and was used for the purpose of this research only.

Study Design and Sample Collection

An HIV-positive/AIDS patients were chosen randomly from the inpatients and outpatients at 1 Brigade Medical Centre, for this study. The patients were informed about the study and their rights to decline or participation in the study was sort. The inclusion criteria are: patients must be HIV-positive, willing to participate in the study and have the ability to comprehend. The exclusion criteria are: HIV negative and inability to comprehend and offer consent to participate in the study. The patients were counseled and verbal consent was obtained before inclusion in the study. HIV-seropositive patients were defined as those who had tested positive for HIV-1 and 2 antibodies using two consecutive rapid test (Abbott, USA) while AIDS patients in addition to being HIV-positive and showing clinical symptoms classified by WHO and CDC as symptoms for AIDS. One hundred (100) HIV seropositive patient's volunteers, whose HIV status was determined before admitted into study.

Specimen Collection

Blood samples were collected into K₃EDTA container and analysed in less than 24hrs. The labelled sample was placed carefully in the Dynal mixer MX1 and allowed to mix gently for 10 mins. A total of 100 samples were obtained from both male and female aged 18–60 years.

Sample/Sampling Technique

The sample for the study were collected from 100 (one hundred) adults' males and females attending the 1 Brigade Medical Centre Sokoto.

Principle of flow cytometry

CD4 Easy Count Cyflow Assay

Principle: The flow cytometric assays work on the principle of light scatter due to different sizes, granularity of the cells passing through the laser beam, and also by the fluorescence emitted by the cells after staining with the specific monoclonal antibodies to cell surface markers that are tagged with different fluorescence dyes. The population of interest can thus be identified and gated. The monoclonal antibodies specifically bind to different surface receptors like CD4 for T helper cells. CD4-PE fluorescence can be analyzed on a Partec flow

Cytometer with an excitation light source of 488nm or 532nm (blue or green solid-state laser). The Cyflow counter is set to counting CD4+T –cell per μ l whole cell as displayed automatically by the instrument software which is controlled by an operator (Fryland et al., 2006).

Procedure:

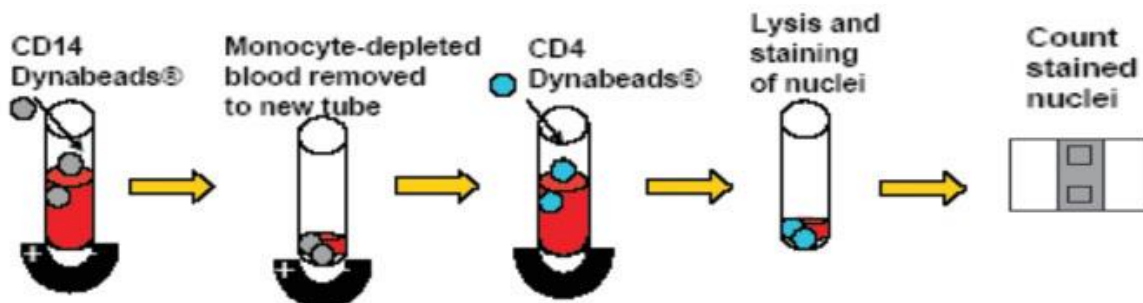
- i. 20 μ l whole blood (EDTA as anticoagulant) was added into a Partec tube.
- ii. 20 μ l of CD4 mAb PE reagent was added to (i) above. Mixed gently and incubated for 15 minutes at room temperature protected from light.
- iii. 800 μ l of no-lyse buffer was added to (ii) and vortexed gently.
- iv. Test sample in (iii) was fed into Partec Cyflow device and reading taken.

Manual CD4 Dynal Bead ASSAY Method

Principle

Immunomagnetic cell isolation using Dynal T4 Quant Kit involves three steps:

- Monocyte depletion
- CD4+ T cells isolation
- CD4+ T Lymphocyte staining and counting



Materials Required

Equipment

- Dynal Sample Mixer: MX1
- Magnet: Dynal Mpctm-5
- Vortex mixer
- Haemocytometer



Dynal Sample Mixer MX1



Magnet: Dynal MPCtm-5

Reagent

Dynabeads are uniform, superparamagnetic polystyrene beads about 4.5 μ l in diameter and coated with mouse antibodies specific for the CD14 antigens of monocytes, or the CD4 antigens of the CD4⁺ T cell subset respectively. The products are suspended in Phosphate buffered saline (PBS) containing 0.1% bovine serum albumin and 0.02% sodium azide

Dynal T4 Quant kit containing:

- Dynabeads CD14
- Dynabeads CD4

- Lysis solution
- PBX BSA 20x concentrated buffer solution

Stains

Acridine Orange staining solution for fluorescence microscopy

- Sternheimer-Malbin staining solution for light microscopy
- Turk staining solution for light microscopy

Materials

- EDTA collecting tubes
- Light or fluorescent Microscope
- Eppendorf tubes

- Cryovials
- Pipettes (25 – 250µl)
- Improved Neubauer Counting Chamber
- Fluorescence or light microscope
- Tally counter
- Sharps container
- Timer
- Permanent marker
- Parafilm
- Beaker
- Pipette tips

Procedure

Cell isolation

- The kit was taken out of the refrigerator and allow getting to room temperature before use.
- 1:20 solution PBX BSA buffer solution in distilled water was prepared.
- The clearly labelled blood sample was placed in the Mixer MX1 at a gentle tilting and rotation for 10 mins.

Cell Isolation

- 2 conical Eppendorf or cryovial was labeled (Rhesus) test tubes with the patients\ identifiers (one for monocyte depletion and the other for CD+4 isolation, lysis and staining.

Monocyte depletion

- With a 1000µl mechanical pipette, 350µl PBX washing dilution buffer was taken.
- The pipette tip *was* wiped with a blotting paper, to avoid the paper come in contact with the pipette tip.
- 350µl PBX washing dilution buffer was placed into the first Eppendorf or cryovial test tube.
- 125µl of well mixed ACD or K3 EDTA blood taken, from the middle of the tube.
- 125µl of blood was taken into the PBX buffer solution already present in the tube, the pipette mixed for three times with the PBX already present in the tube.

- The Dynabeads CD14 was resuspended ensuring that there are no bead clumps in the bottom of the bottle.
- 25µl of Dynabeads CD14 was added to the PBX buffer/blood mixture.
- The cryovials tubes was capped firmly and the mixture mixed gently for 10 seconds.
- The samples were placed in Dynal Mixer and incubated at room temperature with gentle tilting and rotation for 10 mins.
- After incubation, the tube was removed from the mixer and shaken down any blood droplets remaining under the cap.
- The cryovial tubes was taped down to remove any blood that may remain in the cap. The cap or the parafilm was disposed.
- The tube was placed in the MPX magnet for 2 mins to concentrate the beads.
- 200µl of washing/dilution buffer taken and placed into the second labeled tube.

CD4⁺ T cell Isolation

- 200µl of monocyte-depleted blood (supernatant) was transferred into the test tube containing the 200µl of washing/dilution buffer without disturbing the cells held on the tube wall by the magnet.
- The initial tube containing the monocytes was discarded.
- Dynabeads CD4 (yellow label) was resuspended carefully, ensuring no bead clumps in the bottom of the bottle.
- 25µl Dynabeads CD4 was added to the tube containing the PBX- monocyte depleted blood mixture.
- Parafilm was placed on the tube, the tube was Capped and mixed gently for 10 seconds, and Placed in the mixer and incubated with gentle tilting with rotation for 10 mins.
- After the incubation, the tube was removed from the mixer and shaken down any blood droplets remaining under the cap and uncapped.



- The tube was placed in the magnet fMPC-S at room temperature for 2 mins to isolate the bead-bound CD4⁺ T lymphocyte.
- The supernatant was aspirated gently without disturbing the cells attached to the wall of the tube still in the magnet.
- The blood supernatant was removed completely.
- The tube was removed from the magnet.
- 500µl PBX washing/dilution buffer was added to the tube containing the Dyna beads-CD4+ lymphocyte complex.
- Precut paraffin was placed over the tube.
- The tube was rotated a few times for a complete resuspension of the bead-bound cells
- The parafilm was removed and discarded. The tube was placed in the magnet for 2mins to isolate the bead-bound CD4+ T cells.
- The tube was completely removed for the last washing buffer; avoid disturbing the isolated beads magnetized on the tube wall that is the supernatant was pipetted from the opposite side of the magnetized CD4 bead bound cells.

CD4⁺ T lymphocyte lysis and nucleus staining with Turks solution

- The magnet slide was removed from the magnet.
-
- 50µl of lysis solution was taken and the tip wiped with blotting paper.
- The 50µl of lysing solution was added to the Dyna bead –bound T4 lymphocyte complex in the tube.
- The sample vortexed for 10 secs to resuspend the cells.
- The sample was left for 5 minutes at room temperature.
- 50µl Turks staining solution was added.
- Vortexed the sample resuspended for 10 secs.

CD4+ T lymphocyte nucleus counting

- The sample was resuspended by vortexing for 5 secs.
- About 20 µl was transferred to the Haemocytometer and allowed for 2 mins in a moist chamber for the cells to settle.
- The number of cells was Counted using the convectional white light microscope with x 40 objective.
- The purple stained dots (CD4+ T lymphocytes) was Counted in the central large square of 25 x 16 small squares.
- For a Neubauer Cell counter, this formula was used: number of cells/area of the quantification (mm²) x depth of the cell (mm) x the dilution.
- The number of cells counted in the haemocytometer is converted to the concentration of CD4+ T cells in whole blood by the formula:

$$\text{Cells}/\mu\text{l} = \frac{n}{v} \times 2$$

n = number of cells counted

2 = dilution factor

v = volume counted in the haemocytometer (in microlitres). V = Area x depth = 1mm x 0.1

Area counted is 1mm and depth is 0.1mm

$$\text{Cells}/\mu\text{l} = \frac{n}{1 \times 0.1} \times 2 = \frac{n}{0.1} \times 2 = n \times 20$$

Therefore, absolute CD4 cells/µl = no of cells counted x 20

Statistical Analysis

Data collected were entered into IBM compatible microcomputer. Data analysis was done using Statistical Package for Social Science (SPSS) software package. Correlation was by Pearson’s Correlation Coefficient. The Mean and Standard deviation (SD) were computed and results were expressed as mean + SD. Student t-test was used to compare differences between means. Table 1 shows the results precision and reproducibility of CD4 T-lymphocyte count in the samples.

Table 1: Distribution of CD4 value in compares with the Manual method and Cyflow method.

Manual CD4 value	Cyflow value cell/ μ l	Manual value cell/ μ l	Cyflow value cell/ μ l
20	44	260	264
20	44	260	294
60	51	320	360
80	101	380	400
160	200	400	455
160	145	400	350
160	167	400	389
160	180	400	444
160	150	400	381
160	190	400	451
160	151	400	399
160	202	400	390
200	240	400	422
200	244	400	463
200	244	400	380
200	244	400	380
220	243	400	431

**Table 2: Distribution of CD4 value in compares with the Manual method and cyflow method**

Manual CD4 value cell/ μ l	Cyflow value cell/ μ l	Manual value cell/ μ l	Cyflow cell/ μ l
400	450	440	471
400	382	460	504
400	463	500	534
400	470	560	593
400	440	560	602
400	404	600	609
400	380	600	654
400	440	600	622
400	355	600	611
400	400	600	580
400	424	600	620
400	410	600	633
400	408	600	588
400	460	600	560
400	433	600	695
400	433	620	655
400	300	620	602
400	423	640	610
420	444	680	699
420	458	720	737

Table 3: Distribution of CD4 value in compares with the Manual method and Cyflow method

Manual CD4 value cell/ μ l	Cyflow CD4 value cell/ μ l
760	804
760	777
800	900
800	777
800	805
820	833
820	810
820	854
820	844
820	875
840	866
860	888
880	900
900	921
960	1050
980	988
1100	1189
380	400

Table 4: The mean, standard deviation covariance of CD4 value compares the Manual method and Cyflow methods

	Manual CD4 value cell/ μ l	Cyflow CD4 value cell/ μ l
Total	45200	47100
Mean	452	471
STD	246.2	253.1
CV	2.9	8.8

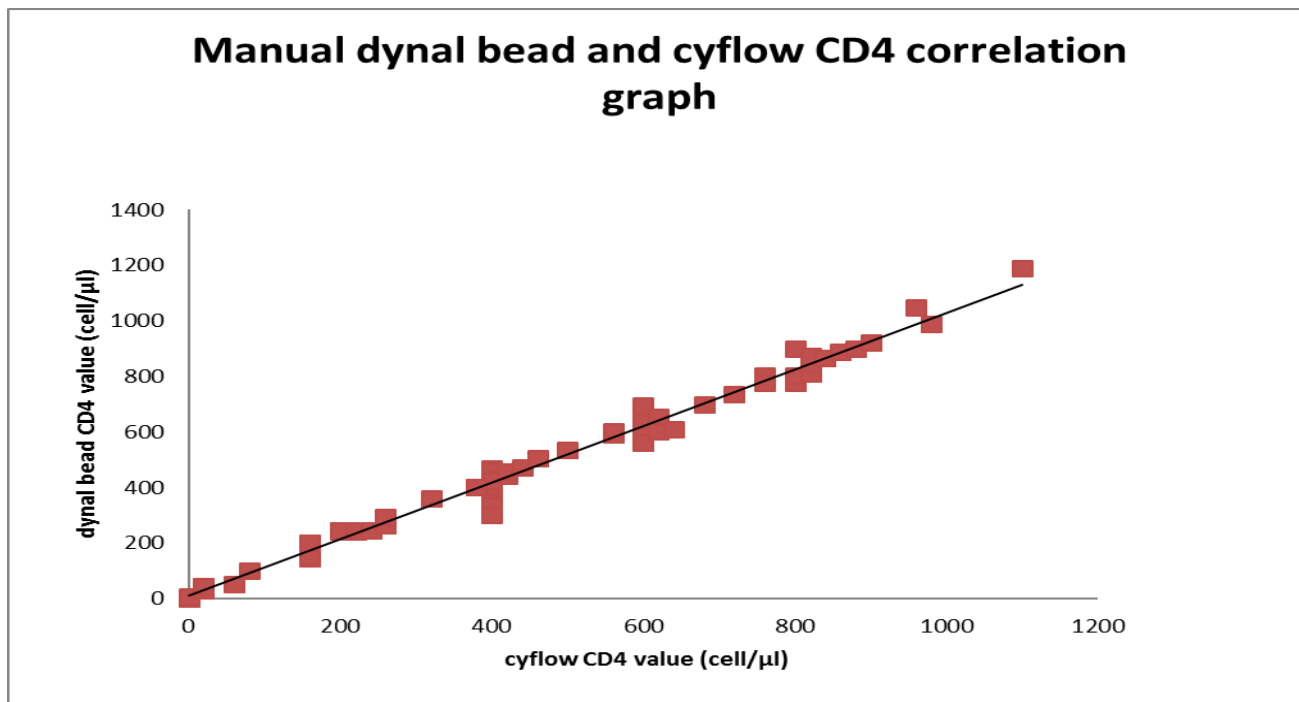


Figure 1: Correlation graph of CD4 values obtained from flow cytometric (Cyflow) count machine and manual Dynalbeads count

Discussion

HIV/AIDS a disease of public health importance has remain the most with it high mortality and morbidity rate especially in developing country like Nigeria. Its management has also pose a medical challenge since it has no permanent cure, hence early detection and management has become the only key to effectively reduce the mortality rate. To achieve the 90:90:90 goal therapy can best be monitored using CD4-T lymphocyte estimation since the virus has it effect on the CD4 T-lymphocyte. The two-major technique Cyflow and Dynalbeads still remain the most effective way of assaying CD4 T-lymphocyte, especially the manual method (Dynalbeads method) the cheapest that can be carried out in the most remote area of the world especially in Nigeria. Hence the comparison of the two techniques, highly simplified, low-cost, true volumetric flow cytometer (Cyflow) with a manual magnetic bead (Dynalbeads) method in a setting of a high HIV-AIDS prevalence rate and had three main findings.

Overall, the two techniques (Dynalbead and Cyflow) produced comparable and well-correlated CD4 T-lymphocyte counts ($n=100$, $p = 0.071$, $r = 0.99$). when compare to ($p = 0.057$, $r = 0.93$) reported by

Godwin et al. (2005). These findings agree with earlier reports which involved the comparison of CD4 T-lymphocyte counts by Dynabeads and other flow cytometry techniques Crowe et al. (2003); Diagbouga et al. (2003) and Lyamuya et al. (1996). Crowe et al. (2003), Diagbouga et al. (2003) and Lyamuya et al. (1996) reports have shown that older generations of flow cytometers, such as those from Becton Dickinson, Coulter, and Ortho Systems, produce reliable and accurate results which are comparable and correlate with results obtained with non-flow cytometric techniques (Dynalbeads). In a study carried out by Crowe and Colleagues (2003), it was found that the Dynalbeads assay was easier to correlate with data from flow cytometry ($n = 54$, $r = 0.96$) than the manual Coulter assay was ($n = 27$, $r = 0.69$). Similar multicenter studies also showed a high correlation ($r = 0.9$ and $r > 0.9$) of flow cytometry (FACS count) with Dynalbeads CD4 T-lymphocyte counts Diagbouga et al. (2003) and Lyamuya et al. (1996).

It is obvious that the optimal design would compare techniques with similar principles and modes of operation (e.g., flow cytometry versus flow cytometry or non-flow cytometry versus non-cytometry techniques); however, in our circumstances and setting as a developing country,

we have chosen primarily to compare the two techniques (a simple, automated Cyflow method versus the manual Dynalbead method) which are the present methods available to us for CD4 T-lymphocyte enumeration to determine which technique would be more cost-effective for CD4 T-lymphocyte estimation in order to meet the overwhelming demand for the test in our center.

The second important result was the finding that Cyflow produced a more precise and reproducible CD4 T-lymphocyte counts than the manual method. The analysis of the CD4 T-lymphocyte data obtained from 20 separate determinations performed on the same sample with the two methods showed CV% of 2.9% for Cyflow and 8.8% for Dynabeads. The marked difference in CV% observed for Dynabeads and Cyflow is not surprising. Manual microscopic techniques suffer limitations in accurately estimating cell counts in a sample with extremes of low and high cell populations compared with automated techniques (Carella *et al.*, 1995). In the low-CD4-T-lymphocyte group, the Cyflow method gave results 10% higher on average than those of the Dynabeads method. Does such a count make a difference? We contend that it does because the absolute CD4 T-lymphocyte count is used as a cutoff point to determine when to administer ARV drugs and also for monitoring immunologic response to ARV drug therapy. This is an exceptionally important consideration in our environment and other developing countries where ARV drugs are scarce.

Our third important finding is that, although the initial investment on purchasing the Cyflow instrument is about 22,000 Euros, it is in the longterm a cheaper, more cost-effective, and more practical method for use in tertiary or referral centers that handle large numbers of blood samples for CD4 T-lymphocyte estimation. As many as 200 samples can be analyzed per day compared to 10 to 15 samples with the Dynabeads techniques (Greve *et al.*, 2003). The cost per test for Cyflow is between \$3.00 and \$5.00, whereas that of Dynabeads ranges from \$12.00 to \$22.00 and other flow cytometry techniques are as high as \$30.00 to \$100.00 per test (Bartholomew *et al.*, 2003). Therefore, Cyflow is three- to fourfold more cost-effective. The Cyflow

instrument, unlike the older generations of flow cytometers, uses one antibody step. The protocol does not require cell lysing, a procedure known to reduce CD4 T-lymphocyte count by 0 to 40% (Gohde *et al.*, 2003; Greve *et al.* 2003) and it has no need for frequent optical alignment by a well-trained operator.

There is no doubt that the manual CD4 T-lymphocyte enumeration techniques have their rightful place, especially in secondary and primary centers where only a few blood samples are tested per day. In these centers, the cost per test can further be reduced by the use of an ordinary light microscope instead of the more expensive and more sensitive UV fluorescence microscope.

In places where the HIV-AIDS burden is high and demand for CD4 T-lymphocyte enumeration is high, the Cyflow technique, which runs as many as 200 samples per day, is cost-effective and ideal. In summary, the various technologies currently available for enumerating CD4+ T-cells are based on distinct principles and have different operational characteristics. New manual technologies based on micro-arrays and dipsticks are being developed and probably will become available in a few years' time. It is also required that laboratories carrying out CD4 lymphocytes count participate in quality control programme to impart reliability to the results. In addition, the lack of ready access to technical support and quality assurance programs has limited the use of flow cytometry techniques in resource-constrained countries.

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References

Balakrishnan, P., Dunne, M., Kumarasamy, N., *et al.* (2004). An inexpensive, simple, and manual



- method of CD4 T-cell quantitation in HIV-infected individuals for use in developing countries. *Journal of Acquired Immune Deficiency Syndrome*; **36**: 1006-1010.
- Bartholomew, C. (2001). Prices of CD4 assays and viral load tests must be reduced for developing countries. *British Medical Journal*; **323**:809b.
- Bi, X., Gatanaga, H., Tanaka, M., et al. (2005). Modified Dynabeads method for enumerating CD4+ T-lymphocyte count for widespread use in resource limited situations. *Journal of Acquired Immune Deficiency Syndrome*; **38**: 1-4.
- Carella, A.V., Miss, M.W., Provost, V. and Quinn, T.C. (1995) A manual bead assay for the determination of absolute CD4+ and CD8+ lymphocyte counts in human immunodeficiency virus-infected individuals. *Clinical Diagnostic Laboratory Immunology*; **2**: 623-625.
- Crowe, S., Turnbull, S., Oelrichs, R. and Dunne, A.(2003) Monitoring of human immunodeficiency virus infection in resource-constrained countries. *Clinical Infectious Diseases*; **37(1)**:S25-35.
- Dagbouga, S., Chazallon, C., Kazatchkine, M., et al. (2003). Successful implementation of a low-cost method for enumerating CD4+ T lymphocytes in resource-limited settings: the ANRS 12-26 study. *AIDS*; **17**: 2201-2208.
- Fryland, M., Chaillet, P., Zachariah, R., et al. (2006) The Partec Cyflow counter for CD4 T cell counting produce high quality results and robust field under routine field condition in Malawi. *Translation of the Royal Society of Tropical Medicine and Hygiene*; **100**: 980–985.
- Godwin E. I., Badung, B., Pam, et al. (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 Diagnostic Laboratory Immunology*; **12(1)**: 224–227.
- Gohde, W., Cassens, U., Lehman, L.G., et al. (2003) Independent patient-dependent influence of erythrocyte lysing procedures on flow-cytometric analysis of leukocyte subpopulations. *Transfusion Medicine and Haemotherapy*; **30**:165-170.
- Greve, B., Beller, C., Cassens, U., et al. (2003). High-grade and hematopoietic progenitor cells caused by erythrocyte-lysing procedures for flow cytometry analyses. *Journal of Haematotherapy and Stem Cell Research*; **12**:321-330.
- Greve, B., Cassens, U., Westerberg, C., et al. (2003). A new no-lyse, no-wash flow-cytometric method for the determination of CD4 T cells in blood samples. *Transfusion Medicine and Haemotherapy*; **30**:8-13.
- Janossy, G., Jani, I. and Gohde, W. (2000). Affordable CD⁺ T cell counts on ‘single-platform’ flow cytometers. I. Primary CD4 gating. *British Journal of Haematology*; **111**:1198-1208.
- Janossy, G., Jani, I. V. and Bradley, N.J. (2002). Affordable CD⁺-T-cell counting by flow cytometry: CD45 gating for volumetric analysis. *Clinical Diagnostic Laboratory Immunology*; **9**:1085-1094.
- Lyamuya, E.F., Kagoma, C., Mbena, E.C., et al. (1996). Evaluation of the FAScount, TRAx CD4 and Dynabead methods for CD4 lymphocyte determination. *Journal of Immunology Methods*; **195**:103-112.
- Nicholson, J.K.A., Velleca, W.M., Jubert, S., et al. (1994) Evaluation of alternative CD4 technologies for the enumeration of CD4 lymphocytes. *Journal of Immunology Methods*; **177**: 43-54.
- Reimann K.A., O’Gorman M.R., Spritzler, J., et al. (2000) Multisite comparison of CD4 and CD8 T-lymphocyte counting by single- versus multiple-platform methodologies: evaluation of Beckman Coulter flow-count fluorospheres and the tetra ONE system. The NIAID DAIDS New Technologies Evaluation Group. *Clinical Diagnostic Laboratory Immunology*; **7(3)**:344-351.
- Schnizlein-Bick, C.T., Spritzler, J., Wilkening, C.L., et al (2000) Site Investigators, and the NIAID DAIDS New Technologies Evaluation Group: Evaluation of True Count absolute-count tubes for determining CD4 and CD8 cell numbers in human immunodeficiency



virus-positive adults. *Clinical Diagnostic Laboratory Immunology*; **7**:336-343.

UNAIDS/World Health Organization. 2003. AIDS epidemic update, UNAIDS/World Health Organization, Geneva, Switzerland.

WHO (2007). Laboratory Guidelines for enumerating CD4 T Lymphocytes in the context of HIV/AIDS. Regional Office for South-East Asia New Delhi.