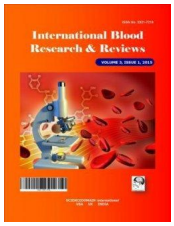

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Suitability of Lower K2 EDTA Sample Volumes for Absolute CD4 Count Enumeration by Flow Cytometric Technique

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Authors' contributions

This work was carried out in collaboration between all authors. Author KAF designed the study, wrote the protocol and wrote the first draft of the manuscript and managed the experimental process. Author ODA managed the literature and statistical analysis. Authors CTO and AJE were also involved in experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Background: Incorrect blood sample volume-anticoagulant ratio has been the cause of both haematological and immunological errors especially when K_3 EDTA-containing blood collection tubes were used. Lower whole blood sample volumes collected into 4.0 millilitres spray-dried K_2 EDTA has been shown to overcome incorrect haematology results when analysed on automated haematology analyzers but there is no experimental evidence for the same in the CD4 count enumeration by flow cytometric technique.
Materials and Methods: 9.0 ml of whole blood was collected from each of fifteen retroviral and ten normal volunteers and aliquot into five different 4.0 ml plastic spray-driedK_2 EDTA blood collection tubes containing 4.0, 2.0, 1.5, 1.0 and 0.5 ml respectively. Each well-mixed sample was analysed on Partec Cyflow counter within 4 hours of collection for absolute CD4+T lymphocyte count.

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Results: Both the reference sample volume 4.0 ml and experimental lower sample volumes (2.0, 1.5, 1.0 and 0.5 mls) of retroviral volunteers in 4.0 ml plastic spray-driedK_2 EDTA blood collection tubes gave comparable CD4 count results with percentage mean difference of 1.82%, -1.48%, 2.25% and 0% for 2.0 ml, 1.5 ml 1.0 ml and 0.5 ml respectively. Irrespective of sample volumes, the normal volunteers had higher CD4 count results. There was no statistically and clinically significant difference in the CD4 counts and the percentage mean difference were 0.4%, 0.17%, 1.00% and 0.23% for 2.0 ml, 1.5 ml, 1.0 ml and 0.5 ml respectively. The correlation (slope)and modest logistic regression coefficient (R^2) of experimental lower sample volumes of both retroviral and normal volunteers were between 0.9500 and 1.0000 showing excellent agreement in the CD4 counts of both reference and experimental sample volumes($p<0.01$).
Conclusion: Quality CD4 count results can be obtained with a minimum sample volume of 0.5 ml in 4.0 ml spray-dried K_2 EDTA vacutainer blood collection tubes both in HIV and healthy individuals with intact immune function.
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Keywords: CD4 count; K_2 EDTA; retroviral volunteers; percentage mean difference; spray-dried cyflow counter.

1. INTRODUCTION

Absolute CD4+ T-lymphocyte count is the diagnostic tool required when considering initiating antiretroviral therapy in newly diagnosed human immunodeficiency virus (HIV) patients as well as for proper monitoring of those already on highly active antiretroviral therapy (HAART). The accuracy and reproducibility of CD4 count results is therefore basic to decision-making in HIV management [1].

Diagnostic medicine like its clinical counterpart is a dynamic scientific world where scientists proffer solution to procedures that pose challenges in the past. One major question that has remained unanswered is whether lower sample volumes collected into spray dried dipotassium ethylene diamine tetra-acetic acid (K_2 EDTA) collection tubes are suitable for flow cytometric analysis of CD4 count. The suitability of lower sample volumes collected into spray dried K_2 EDTA vacutainer tubes for automated haematologic analysis both in normal and pathologic conditions have been demonstrated by American and Nigerian researchers [2,3]. To the best of our ability, there is virtual lack of data on the suitability of same for CD4 count test given the sensitivity of the test, the cost of the risk of misleading the clinicians, toxicological effects of ART on the recipients of the drugs and overall treatment outcome. However, the challenge of having to collect the standard volumes of 4.5 -5.0 ml into K_3 EDTA which is the liquid form of the anticoagulant sometimes pose great challenges to clinicians especially in infants and the elderly as well as highly debilitating patients. Such struggles often times have led to microclots or fully-clotted, haemolysed, non-homogeneous samples and incorrect blood volume – anticoagulant ratio. These more often than not usually lead to rejection of such samples by clinical laboratory scientists.

Absolute CD4+T- lymphocyte count is the determination of the concentration of CD4+T-lymphocytes in the blood. Depletion of the CD4+ T cell has been proved to be responsible for associated immune deficiency in HIV infection [4,5]. It is one of the hallmarks of the progression of HIV infection and a major indicator of the stage of the disease in HIV infected individuals [6,7]. Clinical and Laboratory Standard Institute, CLSI (formerly, NCCLS) in one of her recent publications, 'Procedures for the Handling of Blood Specimens', stated that the amount of additives placed into a tube is intended for certain volume of blood. If less than the required blood volume is drawn, the excess amount of additives has the potential to adversely affect the accuracy of the test [8] and another publication recommended that the draw volume shall be no more than 10% below the stated volume of the manufacturer [9]. These standards apply to all collection tubes including EDTA.

Several published articles have compared the results of samples collected into glass and plastic K3 EDTA, glass K_3 EDTA and plastic K_2 EDTA and have obtained comparable results for full blood count carried out by automated analysers [10-12]. In the last two decades, experts have advocated for the use of spray-dried K_2 EDTA tubes rather than the usual glass K_3 EDTA [13-16,2].

We hypothesized that the spraying of dry K2 EDTA anticoagulant has drastically minimized the immunologic errors due to incorrect anticoagulant – blood volume ratio in just same way it did for haematologic errors.

1.1 The Objective of This Study

We carried out this research to prove our hypothesis that if lower sample volumes collected into spray-dried K2 EDTA gave comparable results with the recommended standard volume (4 ml) in both normal and pathologic samples tested for haematologic parameters, it should do the same for CD4+ T cell count in both normal and pathologic samples; to establish the suitability of analysing absolute CD4 count with flow cytometric method using lower sample volumes, and determine the minimum blood collection volume required for CD4+ T cell count analysis.

2. MATERIALS AND METHODS

2.1 Study Location

This study was carried out at the Haematology department of the Federal Teaching Hospital (FTH), Ido Ekiti, Nigeria. FTH was located in Ido Ekiti, the principal town in Ido Osi local government area of Ekiti state with an estimated population of 107,000. It is geographically located in the northern part of Ekiti state which covers an estimated total area of 6353 km², 2,453 square mile and an estimated population of 2,737,186, where the routes from Kwara and Osun states converge. FTH, Ido Ekiti was upgraded in 2006 to serve as a centre for HIV/AIDS referral, diagnosis and treatment in Ekiti State and serving five contiguous states. The centre since then has been offering free diagnosis and antiretroviral therapy.

2.2 STUDY DESIGN

2.2.1 HIV counselling and testing

All patients newly diagnosed for HIV at our HIV counselling and testing (HCT) site or PEPFAR-supported HIV laboratory (the main laboratory dedicated for confirmation of HIV test results and quality control, and analysis of baseline immunologic, haematologic and other serologic procedures for confirmed HIV/AIDS patients) at the haematology department according to Centre for Disease Control and prevention guideline serial algorithm II were included in the study [17]. Following pre-test counselling and informed consent, we performed HIV testing using two rapid enzyme immunoassay (EIA) techniques. Whole blood samples obtained by capillary puncture or plasma samples separated from 4 millilitres of whole blood collected into K2 EDTA spray-dried collection tubes were used for the procedures and the tests were performed according to CDC-UMD HIV rapid testing serial algorithm II guideline [17]. Similar results were obtained from Genscreen HIV 1 & 2 ELISA kits (Biorad, France).Diagnostic techniques/algorithm was quality controlled using one world Accuracy HIV samples with already known positive and negative HIV results.

2.2.2 Sample collection

Fifteen (15) newly-diagnosed retroviral volunteers were included in the study with ten (10) normal volunteers serving as research controls. 9.0 ml of whole blood sample was drawn from each of the participating volunteers by venepuncture procedure following due pre-test counselling and informed consent as part of ethical consideration of the Federal Teaching Hospital, Ido Ekiti. Each of the donated 9.0 ml blood volume was aliquoted into five 4.0 ml vacutainer blood collection tubes containing K2 EDTA in the following volumes: 4.0, 2.0, 1.5, 1.0 and 0.5 ml.

2.2.3 Cyflow counter calibration/ research sample analysis

Research samples for CD4 count were prepared and run on the Partec cyflow counter (Partec flow cytometer, GMBH, Munster, Germany) according to the manufacturer's instructions. Partec flow cytometer (Cyflow counter) was first calibrated to ascertain optimal equipment performance by using count check beads of already known concentration following daily cleaning procedure. Samples from normal subjects were tested along with research samples to ensure reagent control and quality of results. A well calibrated cyflow counter must give count check beads reagent control within ±10% of reagent concentration. CD4 monoclonal antibodies were used within the expiry dates. Values within ±10% of known results validated the potency of the CD4 monoclonal antibodies used for our research procedure.

2.2.4 Cyflow counter count check beads calculation

A specific count check bead used during analysis of our research samples had known concentration of 23,470 cells/ml. The equipment displayed absolute CD4 count value of the count check bead as 966 cells/µl, and the pre-set dilution factor is 42, then calculated concentration of the count check beads in cells/ml from the flow cytometer

$$= \frac{\text{(CD4 count in cells/µl) X1000}}{42}$$

$$= \frac{966 \times 1000}{42} = 23{,}000\text{cells/ml.}$$

% deviation from the known concentration is calculated from the formula:

% deviation = (23,000-23470)/23470 x 100% = -2.0%.

Since the calculated value of count check beads concentration fell within -10% of known value, the equipment was successfully calibrated.

2.2.5 Principle and procedure of flow cytometry for cd4 count

The cyflow counter operation is based on the simultaneous measurement of multiple physical characteristics of CD4 count in a single file as it flows through the cyflow counter. The counter separated the CD4+ T cell from the monocytes-CD4 bearing cells and noise using a gating system. We prepared the samples and analysed them for CD4 count according to the manufacturer instructions. 20 µl of well-mixed whole blood sample was added to 20 µl of CD4 MAB (monoclonal antibody) in a Rhören tube. This was incubated for 15 minutes in the dark. 800µl of CD4 no-lyse buffer was added (carefully without introducing bubbles) and the mixture was analyzed on Partec cyflow counter and results recorded in cells/µl.

2.3 Statistical Analysis

Data were computed with SPSS statistical software (Statistical Package for Social Sciences Inc, Chicago IL), version 17. The CD4 count results of reference sample volume and lower sample volumes were expressed as means and standards and percentage mean difference using one sample student t-test. The degree of association between the reference CD4 count result (using the standard 4.0 ml sample volume) and the experimental CD4 count results (obtained from lower sample volumes) was further established with Spearman correlation and linear regression analytical tools.

3. RESULTS AND DISCUSSION

9.0 milliliters of blood samples was drawn from each of fifteen (15) retroviral and ten (10) healthy normal volunteers by venepuncture. Each 9.0 ml sample aliquoted into five (5) different 4.0 ml K2 EDTA collection tubes containing 4.0, 2.0, 1.5, 1.0 and 0.5 millilitres samples were analysed on Partec cyflow counter. Mean results, standard deviations and percentage mean difference for absolute CD4 count results of different sample volumes were shown in Tables 1 and 2. The mean CD4 count results for lower sample volumes showed no statistically significant difference from the gold standard 4.0 ml sample volume ($p<0.05$). For the CD4 count results of lower sample volumes (2.0, 1.5, 1.0 and 0.5 ml) to be diagnostically useful, they must give values within ±10% of the gold standard value. Table 1 showed the means and standard deviations of CD4 count results for lower sample volumes of retroviral volunteers to be 439.2±207.5 cells/µl, 447.2±206.7 cells/µl, 432.7±190.5 cells/µl, 449.1± 210.2 cells/µl and 439.2±203.8 cells/µl respectively. The percentage mean differences obtained for 2.0, 1.5, 1.0 and 0.5 ml sample volumes of retroviral volunteers were 1.82%,-1.48%, 2.25% and 0% respectively. The CD4 count results of normal volunteers were higher than the retroviral volunteers irrespective of the sample volumes. This is expected of persons with intact immune status. Table 2 shows the means, standard deviations and percentage mean differences of CD4 count results obtained from the 4.0 ml and for lower sample volumes of normal volunteers. Comparison of mean with t-test shows no clinically significant difference in CD4 count results of 4.0 ml sample volume and 2.0, 1.5, 1.0 and 0.5ml sample volumes. The percentage mean differences for 2.0, 1.5, 1.0 and 0.5 ml sample volumes were 0.40%, 0.17%, 1.00% and 0.23% respectively. The low percentage mean difference greatly reduces the chance of upward misclassification which can lead to delay in antiretroviral therapy initiation or downward misclassification which can prompt treatment decisions.

To further elucidate the diagnostic usefulness and quality of results of lower sample volumes collected into 4.0 ml K_2 EDTA collection tubes,

spearman correlation coefficient and modest linear regression R^2 were used to show the degree of association. Both standard volume and lower sample volumes CD4 count results from retroviral and normal volunteers were excellently correlated since the slope and R^2 of all the sample volumes were between 0.9500 and 1.0000 as shown in Tables 3 and 4.

Blood collection tubes containing K_2 EDTA have gained popularity in the most clinical laboratories in developed countries but the liquid counterpart (K_3 EDTA) is still very much in use in most of our treatment facilities for HIV/AIDS patients in Nigeria. Issues that borders on incorrect blood sample volume – anticoagulant ratio, haemolysis, difficulty in obtaining samples of required volume in exposed babies or geriatric patients and patient assessment requiring that different samples be collected for different tests have all constituted cause of sample rejection by clinical laboratory and the need to collect fresh samples often times based on Clinical and Laboratory Standard Institute guideline [8].

Table 1. Mean ± SD and percentage mean difference of CD4 count values of retroviral samples

Sample volume (millilitre)	Mean±SD (cells/µl)	% Mean difference (%) (Mean CD4 count of GSSV minus mean CD4 count of LSV)/ (Mean CD4 count of GSSV) X 100
4.0	439.2±207.5	-
2.0	447.2±206.7	1.82
1.5	432.7±190.5	-1.48
1.0	449.1±210.2	2.25
0.5	439.2±203.8	-

GSSV: Gold standard sample volume; LSV: Lower sample volume

Table 2. Mean ± SD and percentage mean difference of CD4 count values of normal volunteers (control subjects)

Sample volume (millilitre)	Mean±SD (cells/µl)	% Mean difference (%) (Mean CD4 count of GSSV minus mean CD4 count of LSV)/ (Mean CD4 count of GSSV) X 100
4.0	1193.9±232.7	-
2.0	1198.7±242.2	0.40
1.5	1195.0±244.4	0.17
1.0	1205.8±228.1	1.00
0.5	1196.6±238.4	0.23

GSSV: Gold standard sample volume; LSV: Lower sample volume

Table 3. Correlation and regression analysis comparing the CD4 count results of experimental sample volume of blood collection and 4.0 ml reference sample volume of control (normal volunteers) samples

Sample volume (Milliliter)	Slope of CD4 count values	R^2 of CD4 count values	p-value
2.0	0.9520	0.9940	
1.5	1.0000	0.9830	P = 0.01
1.0	0.9760	0.9830	
0.5	0.9640	0.9690	

Correlation is significant at the 0.01 level (2-tailed)

Table 4. Correlation and regression analysis comparing the CD4 count results of experimental sample volume of blood collection and 4.0 ml reference sample volume of retroviral samples

Sample volume (Milliliter)	Slope of CD4 count values	R^2 of CD4 count values	p-value
2.0	0.9860	0.9910	
1.5	0.9810	0.9900	P = 0.01
1.0	0.9640	0.9890	
0.5	0.9640	0.9920	

Correlation is significant at the 0.01 level (2-tailed)

Our findings based on the use of lower sample volumes in 4.0 millilitres K2 EDTA vacutainer collection tubes revealed the aforementioned problem of incorrect blood sample volume – anticoagulant ratio is not an issue here.

4. CONCLUSION

The study demonstrates that the use of spray-dried K2 EDTA is suitable for CD4 count with a volume of whole blood sample as low as 0.5 millilitre. The statistical analysis shows a high correlation of the small volumes of blood (2.0 millilitres, 1.5 millilitres, 1 millilitre and 0.5 millilitre) with the gold standard volume of 4ml for both abnormal (HIV positive) and normal samples (p=0.01). Standardizing into one 4.0 millilitres spray-dried K2 EDTA tube for vast majority of patients will reduce the need for recollection of samples, simplify pre-analytical testing process, improve staff safety and reduce inventory and supply cost. Where Sysmex KX-21N haematology automated analyser for complete blood analysis and cyflow counter for CD4 count estimation are within the same HIV/AIDS laboratory location, both analyses become highly simplified and patients derive more satisfaction since the minimum sample volume (0.5 millilitre) permits both analyses to be done and gives allowance for repeat of test. Well planned inventory and ordering as well as approval for use is paramount to prevent stock-out and mandatory reverse to old practice. Proper calibration of cyflow cytometer for CD4 count enumeration must also be ensured before analysis for optimal performance and quality results.

INFORMED CONSENT

Pre-test counselling was done and informed consent was obtained from the newly diagnosed HIV individuals before inclusion in the study. Retroviral volunteers already on ART were excluded from the study. No personal bio data was required.

ETHICAL CONSIDERATIONS

CDC guidelines and the ethical guidelines of Federal Teaching Hospital, Ido Ekiti were followed for diagnosis of HIV and inclusion of patients.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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