HEALTH SCIENCES AND DISEASES
The Journal of Medicine and Health Sciences

Original Article

Effects of Storage: Whole Blood Specimens for CD4-T Lymphocytes Determination in Yaoundé, Cameroon

Influence du stockage sur les échantillons de sang total pour la détermination tu taux de lymphocytes T- CD4 à Yaoundé, Cameroun

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Keywords: CD4-T Lymphocytes, Whole blood, Cameroon
Mots Clés : Lymphocytes T - CD4, Sang total, Cameroun

ABSTRACT

Introduction. Stored blood specimens collected in Ethylene Diamine Tetra Acetic Acid (EDTA) anti-coagulant tubes for CD4 T-Cell determinations are inevitable practices in Cameroon and many other countries worldwide. This study aimed to evaluate the reproducibility of results obtained from blood samples stored at room temperature (25° C) and 4° C within six days post collection. **Methods**. Whole blood samples collected in EDTA collection tubes were analyzed for CD4 absolute counts using the FACS Count Machine (Becton Dickinson, France). We Used the FACS Count reagent kit (CD4, CD8 and CD3 combine). Samples were strictly analyzed using the procedural manual of the kit .Freshly collected blood samples in day one served as control. **Results**. There was a significant correlation with results obtained at day 2 and 3 (47 and 72 hours).Day 4, 5, 6 (72, 96 and 120 hours) did not show any correlation with day one. Blood samples stored at -4°C degrees did not correlate with any other day. **Conclusion**. Reproducibility of CD4 cell count could only be evaluated up to 72 hours, whereas blood samples stored in the fridge at -4°C were not reproducible after day one. Our findings can serve as a guide to clinical laboratories and health care providers handling samples in rural and urban settings.

RÉSUMÉ

Introduction. La conservation des échantillons de sang prélevés dans les tubes contenant l'anticoagulant Ethylène Diamine-Tétra-Acétique (EDTA) pour la détermination du taux de lymphocytes T CD4 sont des pratiques courantes au Cameroun et dans de nombreux pays du monde .Cette étude visait à évaluer la reproductibilité des résultats obtenus à partir des échantillons de sang stockés à température ambiante (25°C) dans les six jours suivant la collecte. **Méthodologie**. Les échantillons de sang total collectés dans des tubes EDTA ont été analysés afin de déterminer le nombre absolu de CD4 en utilisant l'appareil FACS Count (Becton Dickinson, France).Nous avons utilisé le kit de réactifs FACS Count (CD4, CD8 et CD3 combinés). Les échantillons ont été analysés en respectant la procédure décrite par le fabriquant du kit. Les échantillons de sang fraîchement prélevés au jour 1 ont servi de témoins. **Résultats**. Nous avons trouvé une corrélation significative entre les résultats obtenus au jour 2 et 3 (47 et 72 heures). Il n'y avait pas de corrélation entre les jours 4, 5, 6 (72, 96 et 120 heures) et le jour un. Les résultats des échantillons de sang stockés à - 4°C n'ont été corrélés avec ceux d'aucun autre jour. **Conclusion**. La reproductibilité du nombre de cellules CD4 ne peut être évaluée que jusqu'à 72 heures, alors que les échantillons de sang stockés dans le réfrigérateur à - 4°C ne sont pas reproductibles après le premier jour. Nos résultats peuvent servir de guide aux laboratoires cliniques et aux prestataires de soins de santé manipulant des échantillons en milieu rural et urbain.

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INTRODUCTION

The measurement of CD4-T cell counts is a widely recommended test for HIV infected patients that provide important information on the immune status of HIV infected individuals[1].The CD4-T cell counts are used as clinical indicators of immune deregulation in individuals infected with HIV and are required for monitoring immune stabilization of T-cells amongst HIV patients on antiretroviral therapy (ART) and prophylaxis for opportunistic infections[2–4].Recently, a UNAIDS Fast-Track targets for 2020, includes achieving major reductions in HIV-related death and incidence cases as per the ouster 90–90–90 targets[5]. This equally include the initiation of ART to all newly HIV diagnosed independent of their CD4- T levels as previously used [1,5,6].Despite these recent recommendations, CD4 still remains an important immunological biomarker in the absence of viral load in managing disease outcomes in resource limited settings[3,4].Clinicians and other healthcare providers are well vested and widely used in the application of CD4-T to determine therapeutic and prophylaxis of opportunistic infections[2]. CD4 test are usually requested as urgent for all newly diagnosed HIV-infected patients within Cameroon[8,9].Procuring accurate reproducible and reliable results for $CD4^+$ T cells counts are therefore vital. There is scarce information available on the reproducibility of whole blood count results from samples stored at room temperature (25° C) or at 4°C in Cameroon. The aim of our study was to evaluate the reproducibility of absolute CD4-T cell count measurements from whole blood specimens stored at room temperature (25° C) or at 4°C tested on days 1, 2, 3, 4, and 6 post collections using the most available and recommended Automated Machine the FACS Count.

METHODS

Study and sample collection

In a cross sectional study, 50 blood speceimen were collected from enrolled HIV-1 positive adult patientsreferred to the Center for the Study and control of Communicable Diseases(CSCCD) of the Faculty of Medicine and Biomedical Sciences of the University of Yaoundé 1, Cameroon for a routine CD4 absolute count measurements.

HIV testing

We used two rapid HIV diagnostic test The Alere Determine™ HIV–1/2 from (Abbott Laboratories, Abbott Park, Illinois, USA) , SD Bioline HIV 1/2 3.0 (Standard Diagnostics, Inc.) and an ELISA antigen antibody kit (Integral Enzynost ELISA kits, Siemens, France) for confirmation. All tests were carried out following the manufacturer's guidelines

Determination of T+ cell counts

Fifty ml of whole blood was collected in a vacutainer EDTA tube using standard blood collection methods. Samples were analysed based on the principle of Immunophenotyping. In brief, 50µl of well mixed whole blood was added to perforated CD4/CD3 and CD8/CD3 reagent tubes containing monoclonal CD4/CD8 antibodies fixed on beads.They were incubated for 80minutes, after which a 50µl of fixative(5% formaldehyde) was added, vortexed and analysed usingthe Fluorescence Activated Cell Sorting (FACS) Count Analyzer (BD FACSCount tri CD4/CD8/CD3 reagent kit) (BD Biosciences, San Jose, California, USA).This procedure was maintained for all 50 blood samples stored at the two different temperatures.All the samples were analysed strictly following the manufacturers' guidelines.

Statistical analysis

Data were entered into a Microsoft Excel spreadsheet and analysed using Version 18.0. Chicago: SPSS Inc Means and standard errors were calculated for all test results obtained. Differences in means were calculated using a two-sample student t test. Data were entered into a Microsoft Excel spreadsheet and analysed using SPSS version 11. The Pearson correlation coefficients were used to determine the relationship between days when the sample was collected and the absolute number of CD4 cells at baseline as measured by the flow cytometer.

RESULTS

Of the 50 samples analysed with day 1 as standard, there were no significants differences between results obtained in 2 days (48hours) and 3 days (72hours) with means of514and 509, respectively. At days 4,5 and 6, there was no significant correlation with day one (398,345,275) as shown in **Table 1(a) and Figure 1**.The Mean and standard deviations were analyzed as shown in table 1 (a) .

Table 1: (a) Summary of statistical analyses

Time	Mean	SD
Day 1	496.0	323.635
Day 2	493.3	324.711
Day 3	490.5	321.643
Day 4	376.3	248.301
Day 5	334.5	237.122
Day 6	256.7	213.803

From Table 1(a) and Figure 1 we observed that the CD4 counts variability reduces over time with a sharp drop in the expected CD4 counts from day 4 onwards. Shown in Table 1(b).

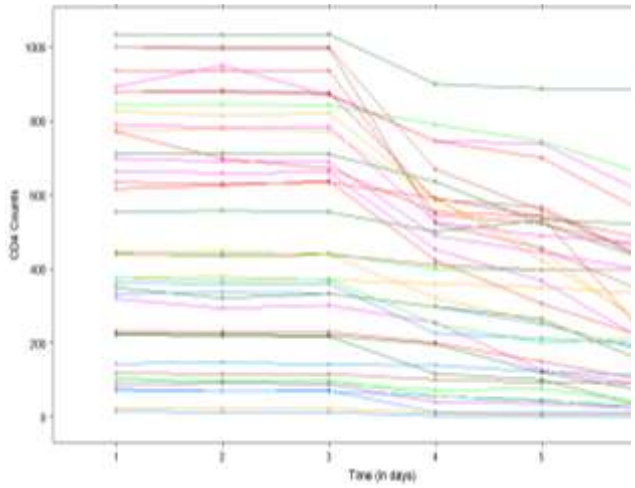

Figure 1: Individual subjects CD4 counts profiles over time. $CD4^+$ T cells were measured each day for a period of 6 days. Room temperature storage was compared to cold storage.

We also observe that the CD4 counts for days 1, 2 and 3 are strongly correlated as compared to counts after day 3 as shown in **Table 1(b)** below

Table 1(b): CD4 counts variability with time

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Day 1	1.00	1.00	1.00	0.95	0.93	0.83
Day 2	1.00	1.00	1.00	0.95	0.93	0.84
Day 3	1.00	1.00	1.00	0.95	0.92	0.83
Day 4	0.95	0.95	0.95	1.00	0.98	0.91
Day 5	0.93	0.93	0.92	0.98	1.00	0.95
Day 6	0.83	0.84	0.83	0.91	0.95	1.00

DISCUSSION

Our results demonstrated that the reproducibility of CD4+T cells count measurement on stored blood samples at room temperature can be evaluated up to 72hours after post collection.Our results vary with that proposed by a study performed from the manufacturers in the USA who attributed 48hr post collection [10], These differences could be attributed to the difference in humidity and temperature in both regions.[10–13]. In another study evaluated in a developing country demonstrated that absolute CD4 lymphocytes determinations on freshly collected blood samples can be analyzed within 4 days (96hours) post collection and produces a reproducible results compared to day one[4,9,14].Our results vary with that obtained from a study carried out by Remoim *et al*., 2004 and Xiar *et al*., 2006 in China and Uganda who demostrated CD4 counts can be valid for 96hr.The differences could be attributed to the method of sample collection they used Heparinated tubes, whereas we worked with EDTA, which is the appropraite anticoagulant used due to it chelating property[3]. It could also be due to the differences in temperature or humidity of different regions [10–13] .

Though many studies have demonstrated that an interval of whole blood storage exceeding 8 hours could causes a significant decrease in cellular immune function[1–3,5,14,16,17], little information is known on the exact storage temperature and could vary from one geographical region to another[4,9,10,16].Regrettably, specimens cannot always be tested on the day of collection, particularlyin the rural areas where thereare no FACSCount machines[3,4,9]. Therefore, specimens have to be transported to nearby laboratories,which are also faced with challenges of periodic shortages of reagent, interruption of electricity supplies, overload with samples and limited staff and equipment breakdown[4,5].Many studies have demonstrated the effect of time and temperature on whole blood stored in EDTA tubes and how its affect lymphocyte viability and phenotype when whole blood is stored overnight at 4°C[10–13,18].

We realize that sample store at 4°C did not give any reproducible results over time and therefore needs to be overlooked.

Recently, muchneeded attention has been given to the conditions under which blood specimens, collected for correlative studies of immune therapy, are handled prior to PBMC isolation[16]. How samples are processed and shipped from trial sites as whole blood or separated PBMC can affect the outcome of immunological monitoring of vaccine-based immunotherapeutic clinical trials[1,5]. Arguably, it is optimal to assay a blood sample immediately and at the site where it is collected, as is done for most routine clinical laboratory tests[1,2]. However, for novel or experimental correlative studies, this is not usually feasible, since expertise for those tests requires specialized laboratories.

CD4+ T- lymphocytes measurement still remains relavant surrogate marker to evaluate immune system restoration in HIV-infected patients and also to cued opportuinistic inffection, a major treat to HIV-infected patients.It is unclear what the future relevance of CD4 counts in the HIV epidemic will be, since everyone will start receiving therapy immediately, regardless of immune status (WHO, 2016).

CONCLUSION

Although it is recommended that sample processing for $CD4^+$ T- lymphoctes cell counts should be done on fresh samples, this is not always possible. Each laboratory should have a standard operating procedure (SOP) for the collection and storage of samples. It is important to keep in mind that the room temperature in one location will be different than the room temperature in the next. For our settings we see that samples can be processed up to 72 hours after collection if kept at room temperature, without compromising the results.

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