Addis Ababa University
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Evaluation of different systems of CD4+ T-cell percentage determinations by using standard flow cytometry for pediatric HIV management

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirement for the Degree of Masters of Science in Biology (Biomedical Sciences)

By

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
APC	Allophycocyanin
ART	Antiretroviral therapy
ARV	Antiretroviral
B-cell	Bone marrow originated cell
B-Lymphocytes	Bone marrow originated lymphocytes
bDNA	branched chain DNA
BLH	Black Lion specialized referral and teaching Hospital
CD	Cluster of Differentiation
CD3	Cluster of Differentiation 3rd molecule
CD4	Cluster of Differentiation 4th molecule
CD45	Cluster of Differentiation 45th molecule
CD8	Cluster of Differentiation 8th molecule
CDC	Center for Disease Control and Prevention
CY5	Cyanin 5
DBS	Dried Blood Spot
DNA	Deoxyribonucleic Acid
DP	Dual Platform
EDTA	EthyleneDiamineTetraacetic Acid
EHNRI	Ethiopian Health and nutrition research institute
FACS	Fluorescence Activated Cell Sorting
FSC	Forward Scatter
FITC	Fluorescein Isothiocyanate
FHAPCO	Federal HIV/AIDS Prevention and Control Office
FMOH	Federal Ministry of Health
HIV	Human Immunodeficiency Virus
LOA	Limit of agreement

LIP	Lymphocyte Interstitial Pneumonia
MTCT	Mother –To-Child-Transmission
NASBA	Nucleic Acid Sequence-Based Amplification
NAT	Nucleic Acid Test
NK-cell	Natural Killer cell
OHL	Oral Hairy Leukoplakia
OI	Opportunistic Infection
p24	Protein-24
PCR	Polymerase Chain Reaction
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PMTCT	Prevention of Mother-To-Child-Transmission
PVL	Plasma Viral Load
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SP	Single Platform
SSC	Side Scatter
T-cell	Thymus derived cells
TLC	Total Lymphocyte count
T-Lymphocyte	Thymus originated Lymphocytes
UNAIDS	United Nations Programme on HIV/AIDS
WBC	White Blood Cell
WHO	World Health Organization
ZMH	Zewditu Memorial Hospital

ABSTRACT

Antiretroviral monitoring relay on %$CD4^+$ T-cell values particularly in pediatrics. The double tube FACSCount system currently in use does not give %$CD4^+$ T-cell value. A single tube FACSCount system that gives %$CD4^+$ T-cell value has been introduced which requires evaluation. These two different systems were evaluated for %$CD4^+$ T-cell measurement by using standard flow cytometery for pediatric HIV management. Blood specimens were collected using K_3EDTA tube from Addis Ababa and its surroundings. Back-calculated %$CD4^+$ T-cells obtained from the double tube FACSCount was compared with %$CD4^+$ T-cells obtained directly from dual platform FACSCalibur Tritest^{TM} system in 221 HIV infected children (less than 14 years). Furthermore, %$CD4^+$ T-cell determination by the newly introduced single tube single platform FACSCount was compared with that of single platform FACSCalibur TruCOUNT^{TM} system in 184 HIV infected subjects (from 1.2 to 66 years). Correlation between the back-calculated %$CD4^+$ T-cells values and %$CD4^+$ T-cell from the dual platform FACSCalibur Tritest^{TM} was good ($r = 0.960$, $p<0.0001$). The Bland-Altman bias plot analysis was +1.6% (with limit of agreement –2.3% to 4.2%; percent similarity 102.2% and coefficient of variation 7.8%). Percentage $CD4^+$ T-cells obtained from the single tube single platform FACSCount has also shown a strong correlation ($r = 0.983$, $p<0.0001$) with the single platform FACSCalibur TruCOUNT^{TM} %$CD4^+$ T-cell values. The bias was +1.7% (with limit of agreement –5.2% to +1.5%; percent similarity 96.5% and coefficient of variation 5.7). In conclusion, correlation between the back-calculated %$CD4^+$ T-cell against the %$CD4^+$ T-cells from dual platform FACSCalibur TriTEST^{TM} and correlation between the new single tube single platform FACSCount system %$CD4^+$ T-cell against that from single platform FACSCalibur TruCOUNT ^{TM} system was strong. Thus, on the basis of strong agreement between the two systems %$CD4^+$ T-cell determination HIV infection in pediatric patients can be monitored by either system.

Key words: Back-calculation, Bland-Altman, CD4 count, Dual-platform, Double tube, FACSCalibur, FACSCount, pediatric HIV, Single platform, Single tube

1 Introduction

1.1 Overview of the HIV/AIDS Pandemic

Since its recognition in 1981 (Gottlieb *et al*., 1981), Human immunodeficiency virus and acquired immunodeficiency syndrome (HIV/AIDS) remains to be the threat of human kind. The global epidemic is stabilizing but still continues to be at unacceptably high level. An estimated 33 million people are reported to be living with HIV/AIDS in 2007 (UNAIDS, 2008). Sub-Saharan Africa, harboring only 10% of the world population, is carrying the highest burden of the infection and is home for approximately 67% of HIV population. In this region, a total of 22 million people are living with the virus and 1.9 million people are estimated to be infected annually (UNAIDS, 2008). In Ethiopia, according to the single point estimate for the prevalence of HIV given by the Federal HIV/AIDS Prevention and Control Office (FHAPCO, 2008), the estimated national HIV prevalence for 2009 is 2.3% with an estimated and number of HIV positive being 1,116,216.

HIV/AIDS remains the leading cause of illness and death in developing countries being exacerbated due to other related conditions such as malnutrition and occurrence of opportunistic infection. Women and children are the most affected groups. Moreover, HIV/AIDS is a major cause of infant and child mortality and morbidity (Gayle & Hill, 2001; ANECCA, 2004), mainly as a result of high rates of maternal HIV infection, high birth rates, lack of access to currently available and feasible interventions and widespread practice of prolonged breast-feeding (ANECCA, 2004).

1.2. Pediatric HIV Infection: Biological mechanism of pediatric HIV infection

Globally about 2.0 million (1.9 million - 2.3 million) children under the age of 15 years are living with HIV. Out of these children, 90% of them live in sub-Saharan countries (UNAIDS, 2008). An estimated 72,945 children live with HIV/AIDS in Ethiopia of which 20,522 need antiretroviral therapy (FHAPCO/FMOH, 2008) and 7399 were receiving antiretroviral therapy as of March 2008 (FHAPCO/FMOH, 2008). This high rate of HIV infection in

children is due to higher infection rate in women of childbearing age (ANECCA, 2004). In Ethiopia, there were 84,189 HIV infected pregnant women and 855,720 orphans in 2009.

More than 90% of children acquire HIV from their mothers. Mother-to-child-transmission (MTCT) of HIV infection occurs both during perinatal or non-perinatal. Perinatal transmission occur intrauterine (during pregnancy), intrapartum (during delivery) and postpartum (during breastfeeding) (Lehman and Farquar, 2007). Other modes of HIV infection in children include through receiving contaminated blood products (Sehgal *et al.,* 2005), being sexually abused (Scarletti, 1996; Sehgal *et al.,* 2005; Lewin, 2007) and during early heterosexual promiscuity (Scarletti, 1996). Although little information is available about the level of MTCT in Ethiopia, a recent report indicated that more than 30% of HIV infection in children is due to breast feeding (FMOH, 2008).

Intrauterine HIV infection is defined as detection of HIV in the blood of infants by DNA-PCR within 48 hours of life (Mahmoudi, 2001). This type of infection is suggested to occur when there are breaks in the placenta barrier or when there is cell-associated viral transcytosis (Hocini and Bomsel, 1999), which gives the virus a chance to cross placenta. In this case, maternal infected blood admixes with fetal blood, which may allow the virus to infect the fetus. Normally placenta is resistant to HIV (Briars *et al.,* 2004), but during transcytosis, infected maternal cell might transport into fetal environment allowing infection.

In intrapartum infection (i.e., infection during delivery) the child is negative for DNA-PCR during the first week of life. However, a negative result does not mean that the infant is not infected but rather exposed, since infection could occur late during pregnancy (Mahmoudi, 2001). During delivery as the neonate passes through the birth canal, it is exposed to infected maternal blood and genital fluid (Lehman and Farquar, 2007). Supportively a more maternal HIV-1 DNA in cord blood of infected infants was demonstrated suggesting intrapartum mother-to-child-transmission associated with placental microtransfusions (Kwiek *et al.,* 2008).

Likewise, in postpartum infection, infants will be infected during breastfeeding. This has been demonstrated in infants in whom the virus was detected after 3 months by DNA PCR but not in the first three months (Wilfert *et al.,* 1994). The most likely mechanism of entry of the virus is the gut mucosal surface (John-Stewart *et al.,* 2004) or through tonsil (Rousseau *et al.,* 2004), by a mechanism of penetration of submucosa in the presence of mucosal breaches or lesions, or via transcytosis (WHO/UNICEIF, 2004). Infant tonsils are suggested to be a portal of entry for HIV-1 in breast-milk transmission (Baba *et al.,* 1994) as has been demonstrated in a macaque Simian Immunodeficiency Virus model.

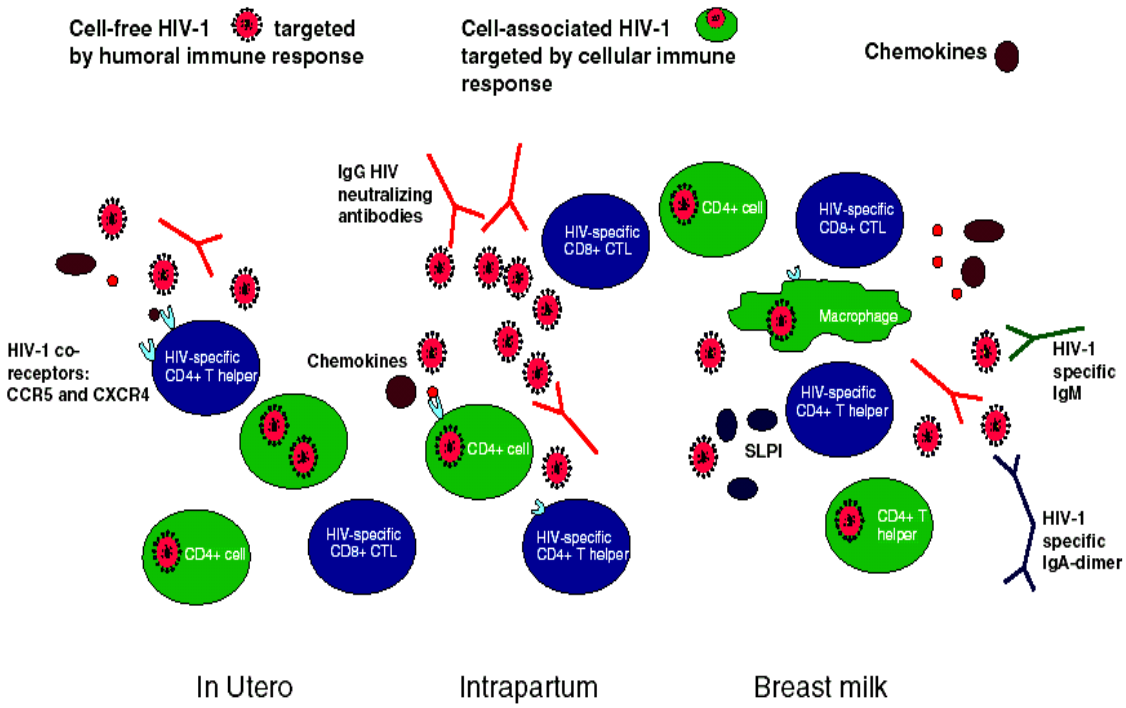


Figure 1. The two arms of immune responses targeting the cell-free and cell-associated viruses during pregnancy, delivery and breastfeeding (Lehman and Farquhar, 2007).

There are certain factors that facilitate MTCT of HIV infection. These include high maternal viral load in peripheral blood (Dickover *et al.,* 1996; Sperling *et al.,* 1996; Douglas *et al.,* 1998) as well as in genital tract (Coombs *et al.,* 2001; Adal *et al.,* 2005; John-Stewart *et al.,*

2005), low maternal CD4 count (Dickover *et al.,* 1996; Sperling *et al.,* 1996; Douglas *et al.,* 1998), clinical stage of the mother especially during delivery and any onset of opportunistic infections related to sexually transmitted disease, prolonged labor (Garcia-Tejedor *et al.,* 2003), breastfeeding (John-Stewart *et al.,* 2004) and also malaria increases MTCT in Africa (Brahmbhatt *et al.,* 2008).

Despite the aforementioned factors that facilitate MTCT of HIV infection, some infants born to HIV-infected mothers avoid HIV infection due to spontaneous HIV clearance or transient HIV infection or sometimes through a mechanism explained as seroreversion (Bryson *et al.,* 1995; Bongretz, 2001). This is due to immune responses generated by the infant which might contribute in protection against HIV-1 (Farquhar and John-Stewart, 2003). Neutralizing antibodies and HIV specific cytotoxic T-lymphocytes, the two arms of immune responses, are fundamental for a protective immune response to HIV. Recently neutralizing antibodies of young infants was identified and these antibodies are capable of challenging HIV antigens. However, due to continuous evolution of virus variants, still there is a chance that HIV escapes the effect of neutralizing antibodies (Geffin *et al.,* 2003). Figure 1 explains both arms of immune systems that are critical in prevention of HIV-1 infection (Lehman and Farquhar, 2007).

1.3. Pediatric HIV prevention and treatment

The significant source of HIV infection in infants is infected mothers. Thus, prevention strategies would be the reduction of mother-to-child-transmission. Mortality in infants due to HIV/AIDS can be reduced by (i) identification of HIV-exposed infants and HIV-infected women during pregnancy (Working group, 2008), (ii) diagnosis of newborns and perinatal and newborn antiretroviral therapy (Working group, 2008), (iii) offering appropriate care, management and monitoring by identifying antiretroviral therapy (ART) eligible children and offering prophylaxis in association with clinical and immunological abnormalities (WHO, 2006) and (iv) providing aggressive optimal nutritional support that could delay the disease progression (Anabwani and Navario, 2005; WHO, 2006).

Intervention strategies for HIV infected children focus on both ART and non-ART methods. Antiretroviral therapy is not an emergency intervention strategy to use in HIV infected children. Prior to ART HIV exposed and/or infected infants and children need regular follow up and assessment of health in order to ensure their eligibility for treatment (FMOH, 2008). To support ART-based intervention, World Health Organization (WHO) has set certain criteria for initiation of ART in infants and children, which are also adopted by Ethiopia, are shown in Annex 1 (FMOH 2008). The antiretroviral regimens so far available for HIV infected children globally and nationally are described in Annex 2.

1.3.1. Health assessment of HIV infected pediatric patients

Health assessment for HIV infected children is given before ART treatment. Pre-treatment health assessments include history of the child, nutrition and growth (weight, length/height and head circumference) assessment, developmental (neurodevelopmental) assessment, clinical assessment, laboratory (complete blood count, CD4 count and viral load), tuberculosis risk assessment, contrimoxazole preventive therapy, immunization, adherence and disclosure. HIV-exposed children who are asymptomatic should usually be assessed initially at every 3-6 months (FMOH, 2008) then every three months until 18 months of age. HIV-exposed infants, who are symptomatic, have poor growth, failure to thrive or recurrent illnesses should have more frequently since the rate of disease progression in these children is rapid. Table 1 shows details in health assessment.

Table 1. Initial and follow up visit assessment reviews (Ethiopia Federal HIV/AIDS Prevention and Control Office Federal Ministry of Health, July 2008).

Clinical assessment of the HIV-infected child should focus on the following:

History – with particular emphasis on previous AIDS defining conditions, history of ARV exposure (previous antiretroviral therapy), family members who are aware of the diagnosis, parental concerns, intercurrent illness

Nutrition and growth assessment – plot weight, height and head circumference on the growth chart

Developmental assessment – developmental signs using reference provided (Harriet Lane Handbook of Paediatrics)

Detailed physical exam – looking for symptoms and signs suggestive of severe HIV infection

Clinical staging – using WHO clinical staging should be done every visit (Annex 3)

Immunological staging – using WHO immunological classification (Annex 4)

Tuberculosis risk assessment – ask about history of tuberculosis contact, tuberculin skin test for tuberculosis screening, and chest radiograph if clinically indicated

Cotrimoxazole preventive therapy – ensure eligibility based on clinical stage and or CD4 if available to provide preventive therapy

Immunizations – according to the National Expanded Program on Immunization

Nutrition counseling – on provision of adequate nutrition, offer support as necessary

ART eligibility – should be reviewed based on clinical stage and immunological criteria

Adherence – to care and cotrimoxazole preventive therapy should be reinforced at each visit

Disclosure – of HIV status to a child should be discussed with the caregiver. Disclosure should be introduced early on in a neutral way, and should be tailored to the developmental maturity of the child. It is particularly important that adolescents be informed of their status so they can become active participants in their own care

Monitoring and evaluation – complete all pediatric intake forms.

1.3.2. Diagnostic Methods of HIV Infection in children

Identification of HIV exposed and infected infants and children is the vital step for care, management, treatment and monitoring in pediatric HIV prevention. The rational approach for the diagnosis of HIV infection in children is using both clinical and laboratory-based methods. WHO has developed algorithms (Working group, 2008) for diagnosis of HIV infected children that are already adopted in Ethiopia are being used both internationally and nationally (Annex 5a & b) (FMOH, 2008).

1.3.2.1. Presumptive/Clinical diagnosis

A presumptive diagnosis is usually based upon the clinical condition of the child and determined mainly for the purpose of providing medical care to the child. It is mostly utilized for those children severely harmed by the infection requiring frequent and close follow up. In areas where there are no proper confirmatory tests, children are diagnosed by any AIDS-indicator conditions such as oral thrush, severe pneumonia and severe sepsis.

In 1980's WHO has developed a clinical case definition and staging system for HIV infection (PAHO, 1989). In later years, together with United Nations Children's Fund, WHO developed a strategy (integrated management of childhood illness) to provide guidelines for the diagnosis and management of sick children (WHO, 1995). However, based on evaluations on clinical staging systems in sub-Sahara African children, particularly infants, the guideline has limited sensitivity (Jones *et al.,* 2005). Therefore, a revised case definition of HIV-1 infection for surveillance purpose and a revised clinical staging classification of HIV-1 related disease in adults and children (WHO, 2006) was developed that include the clinical criteria for the presumptive diagnosis of severe HIV-1 disease to allow early initiation of ART. There are four clinical criteria or staging (asymptomatic-stage I, mild-stage II, advanced-stage III and severe- stage IV) that have been developed for the defined clinical conditions. Based on the clinical case definition and staging system that WHO has revised, Ethiopia has developed clinical criteria for presumptive diagnosis (FMOH, 2008) in severe HIV-1 infected infants and children <18 months of age (Annex 6).

1.3.2.2. Laboratory-Based /Definitive /diagnosis

A definitive diagnosis of HIV infection bases upon laboratory methods (tests), which identifies or confirms the presence of anti-HIV antibody and HIV viron in the blood (Scott, 1994; Chantry *et al.,* 1995). The tests can be performed using two methodologies: the serologic and virologic tests. Serologic test detects the antibody response to HIV, while virologic assays detect and quantitate the genetic material and components of the virus. For a better child care and treatment, developed countries perform a complete definitive diagnosis that includes immunological, virological and viral culture methods. Due to limited facilities, trained human resources and lack of adequate child care and treatment facilities in developing countries, particularly in sub-Sahara African region, performing complete diagnosis is a persistent problem (Tawfik and Kinoti 2003).

Virtually all infants born to HIV infected mothers are seropositive to antibody tests due to passive transfer of maternal antibodies across the placenta, which will continue to be positive for about 12 months due to the omnipresence of maternal anti-HIV antibody. That does not mean that the child is infected but rather explains the child is exposed to HIV infection. To rule out this type of controversies DNA polymerase chain reaction (PCR) test is crucially important. Antibody test is useful when the child reaches 18 months of age and above that the time of actual antibody is developing.

DNA PCR test, which is the preferred and standard virologic diagnostic assay for infants who are born to HIV infected mothers have been used in many countries (Richardson *et al.,* 2008; Creek *et al.,* 2008; Jacob *et al.,* 2008). The Ethiopian pediatric guideline recommended that DNA PCR assay should be performed at 14–21 days of age, at age of 1–2 months, and finally the infant will be retested at age of 4–6 months for definitive exclusion of HIV infection (FMOH, 2008). However, in current situation of the Ethiopian context, the assay is performed at 6 weeks of age or at the first opportunity thereafter (FMOH, 2008). Infants with positive test results are presumably infected and should be referred to care and treatment. The test utilizes whole blood (Piwowar-Manning *et al.,* 2008) and/or dried blood spots (DBS)

(Cassol *et al.,* 1996) on a filter paper. Utilization of DBS has an advantage of minimized biohazard risk and can be stored at room temperature for a while or at-20 to -80^0C for a longer period (Cassol *et al.,* 1996). One disadvantage of DNA-PCR test is its technology requirement with trained human resource.

The other diagnostic method, which is also useful for the diagnosis of infant HIV infection, is identification of p24 antigen (WHO, 2006). The test is an ELISA-based technology and less expensive than nucleic acid tests (DNA/RNA PCR), however, the equipment and its maintenance and the required consumables are not commercially available together (Read *et al.,* 2008). A study done in Uganda (an African experience) showed that p24 antigen detection is as sensitive as required for the determination of infant HIV-1 infection in HIV-1 infected children tested in the first two years of life (Guay *et al.,* 2000) but it does not correlate with lack of HIV-1 infection.

1.3.3. Pediatric HIV Monitoring and Antiretroviral management

1.3.3.1. Clinical monitoring

Following identification of HIV infection, children should undergo a baseline clinical assessment in order to determine the clinical stage of HIV disease. Clinical monitoring provides information on clinical conditions of prognostic significance. It also helps to classify the course of HIV infection into four stages using the WHO clinical staging system (WHO, 2008) (Annex 3). After classifying their clinical staging, identification of associated medical conditions (P*neumocystis carinii pneumonia* (PCP) and tuberculosis) is useful to reduce morbidity and mortality. After infected children are in the treatment continuum they are guided to decisions on when to start cotrimoxazole preventive therapy and, ART and other HIV-related interventions (WHO, 2006).

Based on their status, both pre-ART and on ART, close clinical and laboratory monitoring – in every follow up visit should be scheduled. Pre-ART children should be monitored every 3-6 months (Working group, 2008). The Ethiopian Pediatric Guideline for HIV care and

treatment recommended the schedule for the follow up for pre-ART children, starts at the age of 0-12 months (followed monthly), at the age of 12-24 months (followed monthly) and at the age of 24 and above months (followed every 2-3 months) shown in Annex 7 (FMOH, 2008). But for HIV infected children on ART follow up schedules are different, initially 2-4 weeks prior to antiretroviral therapy, children undergo an initial assessment then the first visit will take place two weeks after initiating therapy and the second and third visits are after 4 to 8 weeks until the child stabilized the treatment. However, once they stabilized the treatment, they can be monitored every 2-3 months Annex 7 *(*FMOH, 2008*).* Although clinical monitoring remains the most essential basis, it lacks sensitivity in determining both disease stage and progression and is thus used in conjunction with laboratory measures.

1.3.3.2. Virological Monitoring

Plasma viral load (PVL) level predicts disease progression and mortality in HIV-1-infected children (Shearer *et al.,* 1997). High level of plasma viral load in adults characterizes acute HIV infection; on the contrary it is the opposite in children since HIV RNA copy number initially raises to high peak level, as high as 10^5-10^7 copies/milliliter in vertically-infected infants during the first 1-2 months of age (Shearer *et al.,* 1997). After this initial peak PVL decreases gradually during the first two years, reaching 34,000 copies/ml and continues declining at a rate of mean log of 0.2-0.3 per year up to the age 6 (Shearer *et al.,* 1997). There are reports indicating a prolonged elevation of PVL level in infants and early childhood due to a larger pool of lymphocytes particularly $CD4^+$ T-lymphocytes resulting from active thymopoiesis. Additionally due to reduced or inability of immune responses (specifically any effective virus-specific immune responses) production may contribute to increasing viral load. However, as the children get older the immune system starts developing resulting dropping of the viral load gradually (Shearer *et al.,* 1997). Quantitative HIV-1 RNA test detects and quantifies the level of HIV-1 RNA in plasma and serum (Glass, 2006), which determine the viral setpoint to decide when to initiate ART and to indicate viral response to therapy as well as virologic failure (WHO, 2006).

Laboratory monitoring of HIV infected children requires disease staging using quantitation of PVL and determination of $CD4^+$ T-cells. Monitoring using PVL in addition to $CD4^+$ T-cell count improves assessment of ART (Hughes *et al.,* 1997) even though they are negatively correlates and independently predict clinical course of HIV-infection in infants and children (Palumbo *et al.,* 1999). To get better clinical pictures of HIV infected children, viral load assays must be combined with $CD4^+$ T-cell count.

1.3.3.3. Immunological Monitoring

Cellular components of blood comprise red blood cells, white blood cells and platelets. White blood cells consist of granulocytes and agranulocytes (monocytes and lymphocytes). Of the white blood cells lymphocytes are morphologically heterogeneous immune cells that have different surface markers (membrane proteins), which will provide information to identify different subpopulations. This property serves as a phenotypic marker, by giving lymphocytes a nomenclature of cluster of differentiation (CD) followed by number of subtypes (CD1, CD2, CD3, CD4, etc...). CD markers are a group of special molecules on the surface of the cells in our body, which is monomeric transmembrane glycoprotein (Dalgleish *et al.,* 1984; Klatzmann *et al.,* 1984). So far more than 300 CD molecules have been identified (Zola, 2006) that would help to identify cell surface antigens that can be distinguished by monoclonal antibodies.

Enumerating the number of different blood cell types is an important diagnostic tool especially in the era of AIDS. For example, determining the number of white blood cells in a blood sample provides an indication of infection; determining the number of platelets, red blood cells and other hematopoietic system components (including reticulocytes) also provides information on the status of a patient. More recently due to the increase incidence of HIV/AIDS, counting of a specific population of leukocytes such as $CD4^+$ T-cells has become extremely important. $CD4^+$ T-cells are subtype of T-lymphocyte which play a major role in orchestrating the immune system by providing help, and augmenting cell mediated immune responses. The mechanism of HIV infection, which results AIDS, is mediated via the binding

of HIV to $CD4^+$ T-cells. Infection of $CD4^+$ T-cells leads to destruction of $CD4^+$ T-cells through different mechanisms including, direct cytophatic effect of the virus, syncytia formation and higher turnover of cells due to immune activation resulting in deterioration of immune responses (McCune, 2001). Thus, enumeration of $CD4^+$ T-cells helps to estimate disease progression and the extent of immune system deterioration.

$CD4^+$ T-cell levels in healthy HIV-uninfected individuals can be affected by certain factors such as age (Tsegaye *et al.,* 2003; Bartlett and Gallent, 2003), gender (Foca *et al.,* 2006; Ray *et al.,* 2006), race (Bussmann *et al.,* 2004; Aina *et al.,* 2005), geographical location (Kassu *et al.,* 2001), diurnal variation (Carmichael and Abayomi 2006), physical exercise and other environmental factors (Worku *et al*.,1997, Tsegaye *et al.,* 1999, Messele *et al.,* 1999, Kassu *et al.,* 2001, Tsegaye *et al.,* 2003). Different studies showed changes in $CD4^+$ T-cell level in peripheral blood with age particularly in children (Denny *et al.,* 1992; Assawawitoontip *et al*., 2003). Lymphocyte profile of healthy, HIV-non-infected children is different from adults. A study conducted on Ethiopian newborns showed higher lymphocyte population in cord blood which declined with age (Tsegaye *et al.,* 2003). In addition, several studies have demonstrated absolute $CD4^+$ T-cell count varies within an individual child with age (Bartlett and Gallent, 2003) while $\%CD4^+$ T-cells relatively remain constant. As a result HIV/AIDS care and treatment guidelines recommend the use of $\%CD4^+$ T-cells count together with absolute counts up to the age of five (WHO 2006, FHAPCO/MOH 2008).

$CD4^+$ T-cells enumeration

$CD4^+$ T-cell enumeration is carried out by immunophenotyping technique and they are reported as the number of cells per cubic millimeter or microliter of blood. Absolute $CD4^+$ T-cell count is the number of $CD4^+$ T-lymphocytes circulating in the peripheral blood, while $\%CD4^+$ T-cell is the proportion of $CD4^+$ lymphocyte among all lymphocyte that are found in the blood (Bartlett and Gallent, 2003). In healthy HIV-non-infected adults the normal level of absolute $CD4^+$ and $\%CD4^+$ T-cell count is 600-1500 cells/mm^3 and 40%, respectively (Bartlett and Gallent, 2003). The reported absolute reference value is much lower for

Ethiopians, 366-1235/uL (Tsegaye *et al.,* 1999). The normal values of $CD4^+$ T-cell counts in young healthy children (less than 5 years old) are higher than adults, varying from around 3,000/mm^3 at 6 months of age up to 2,500/mm^3 and 1,500/mm^3, respectively, at 12 and 24 months of age (Erkeller-Yuksel *et al.,* 1992). The reported median values for Ethiopians are 1816 cells/μL for neonates, 1596 cells/μL for under five children, 942 cells/μL in children aged 5-16 years and 761 cells/μL for adults (Tsegaye *et al.,* 1999; Tsegaye *et al*., 2003). Immunophenotyping technique is used (CDC, 2003) to enumerate these cells.

The absolute number of $CD4^+$ T-cells can be enumerated using the non-flow cytometer (manual) and flow cytometer method. A manual bead assay, called the cytosphere assay, can enumerate $CD4^+$ T-lymphocyte without flow cytometer. An inert latex sphere coated with monoclonal antibody used to identify and manually enumerate the absolute count of $CD4^+$ T-lymphocytes using haemocytometer and visible light microscopy (Carella *et al.,* 1995; Balakrishnan *et al.,* 2004). Some laboratories are still using the manual technique (Nouanthong *et al.,* 2006). However, its disadvantage is time consuming and large number of samples cannot be done in short time.

Flow cytometer is an instrument used for counting and sorting of $CD4^+$ T cells while they are suspended in a stream of fluid and passing by through a focused laser beam. It is the most accepted standard method for enumeration of $CD4^+$ T-lymphocytes because of its accuracy, precision and reproducibility. Currently the flow cytometer technique provides dual platform (DP) or single platform (SP) approaches. The DP approach uses two instruments to generate absolute $CD4^+$ T-cell count: a flow cytometer for generating $\%CD4^+$ T-cell among lymphocytes and a hematology analyzer to enumerate total lymphocyte count (TLC) (Calvelli *et al*., 2003). An absolute count is then derived by multiplying $\%CD4^+$ T-cells by total lymphocyte count, which means DP requires the result of three separate laboratory tests $\%CD4^+$ T-cell (from flow cytometer), total white blood cell (WBC) count and percentage lymphocyte (from hematology analyzer). The SP approaches provides absolute $CD4^+$ T-cell count directly from flow cytometer (Calvelli *et al*., 2003).

Absolute $CD4^+$ T-cell values can be determined using several SP available in the market (Piątosa, 2007). One of the commonly used platforms in this category is the FACSCount system, which is the commonest platform in resource-limited settings (van de Perre *et al.*, 1998). However, the FACSCount system on its own, does not give a $\%CD4^+$ T-cell count (Deems *et al.*, 2004). Therefore, determination of $\%CD4^+$ T-cell in laboratories having the existing SP FACSCount system requires a DP system, in which the total lymphocyte (TLC) will be derived from the hematological analyzer, and the absolute $CD4^+$ T-cell count from the FACSCount and the percentage is back calculated from the absolute $CD4^+$ T-cell count and TLC.

On the other hand both absolute $CD4^+$ and $\%CD4^+$ T-cells can be determined in SP or DP format using the FACACalibur system which is the gold standard (Colombo *et al.*, 2008). However the utilization of FACSCalibur system is limited due to the presence of very limited number of facilities with FACSCalibur systems, the need to have trained human resource and the expensive initial cost of the instrument in the developing world. As a result the ART services in these countries use the FACSCount system to enumerate $CD4^+$ T-cells for initiation and monitoring of ART both in adults and pediatrics knowing that the existing double tube FACSCount system is a limitation for pediatric use for monitoring of ART. Considering the inherent problems of the existing double tube SP FACSCount system for the determination of $\%CD4^+$ T-cells, more recently Becton Dickinson has developed a new single tube FACSCount system that enables the enumeration of $\%CD4^+$ T-cells in SP format like that of FACSCalibur (CDC, 2003; Servais *et al.*, 2004) which simultaneously measures both absolute $CD4^+$ and the $\%CD4^+$ T-cells. Apart from these flow cytometers that developed by Becton Dickinson, there are number of emerging flow cytometry instrumentations developed by other companies such as Point-of-Care, Pima, Labnow etc. that enumerated absolute and $\%CD4^+$ T-cells.

1.4 Justification of the study

As of 2009, more than 441 actively ART sites are operational in Ethiopia (AIDS resource center, 2009) and 118 hospitals and 8 regional laboratories have facilities for laboratory

based monitoring. Despite extensive expansion of care and treatment and progress in ART monitoring of HIV infected adults, pediatric HIV/AIDS management remains severely limited. The two most commonly available and used flow cytometry machines in Ethiopia for ART initiation and monitoring are FACSCount, which is available in all ART laboratories, and FACSCalibur (gold standard) that is available at a referral level in the regional laboratories only. FACSCalibur gives both absolute and %$CD4^+$ T-cell counts while the existing double tube FACSCount system is producing absolute $CD4^+$ T-cell count only. But, in pediatrics ART management absolute $CD4^+$ T-cell count is not as reliable as %$CD4^+$ T-cell value since the absolute value varies with age. As a result WHO recommended the use of %$CD4^+$ T-cell levels for pediatric HIV management (WHO, 2006). The limitation of the existing double tube SP FACSCount system, for not providing %$CD4^+$ T-cell count, was one of the challenges in pediatric HIV monitoring in resource limited settings. As a result, clinicians in Ethiopia have been using the back-calculation method to obtain %$CD4^+$ T-cell values although the clinical reference of the method has not been documented. For the utilization of back-calculated % $CD4^+$ T-cell in Ethiopia it is useful to compare it against the gold standard flow cytometry which is currently in use in Ethiopia. Thus the present study aims to compare %$CD4^+$ T-cell s obtained by back-calculation from FACSCount to the %$CD4^+$ T-cell from FACSCalibur directly.

Moreover, cognizant of the inherent problems of the existing double tube SP FACSCount system for pediatric HIV management, Becton Dickinson Biosciences (BDB) has begun producing a new single tube SP FACSCount system which can be used for the enumeration of both %$CD4^+$ and absolute $CD4^+$ T-cell count at the same time. Formal evaluation of the new FACSCount system is needed before rolling out for widespread use in the country. If this study proves successful, the access to initiate and monitor ART for children would improve dramatically. Here it was hypothesized that both the back calculated %$CD4^+$ T-cell values currently in use by pediatric ART physicians in the country and the new system, which is recently introduced by BDB, but not yet distributed in ART laboratories in Ethiopia, will have good agreement with the gold standard FACSCalibur system.

2 Objectives

2.1. General objective

The general objective of this study is to evaluate different systems of $CD4^+$ T-cell percentage measurements by using standard flow cytometer for pediatric HIV management.

2.2. Specific objectives

- To evaluate $\%CD4^+$ T-cells obtained from the existing double tube FACSCount by back-calculation and compare with the values obtained from DP FACSCalibur (TriTEST^{TM}), and to see whether the results of the two systems agreed well to be used interchangeably
- To evaluate the new single tube SP FACSCount $\%CD4^+$ T-cell determination system and compare it to the gold standards DP FACSCalibur (TruCOUNT^{TM}) system.

3. Subjects and Methods

3.1. Study design

This study was a cross-sectional study performed in and around Addis Ababa ART centers. The study subjects were selected by using a consecutive sampling technique with an unlinked and anonymous data management.

3.2. Study Area and Subjects

There were two groups of subjects involved in this evaluation study during the period July 2007 to April 2008. The first group include 221 HIV infected children ranging in age from 9 months to 14 years attending pediatric ART clinics in two hospitals in Addis Ababa Black Lion specialized referral and teaching hospital (BLH) and Zewditu Memorial Hospital (ZMH). Among these, 169 were from BLH while 52 were from ZMH. For each child included in the study, leftover blood samples were collected from routine laboratory services. Demographic and some clinical variables were collected from patient records. The leftover blood samples were used for evaluating the back calculated % $CD4^+$ T-cell against that directly obtained from the FACSCalibur (plain tube TriTEST^{TM}). The second group of subjects were 184 HIV infected subjects (pediatrics and adults) ranging in age from 1.2 to 66 years, referred to EHNRI national HIV laboratory from sites with no or failed FACSCount facility from in and around Addis Ababa (Sebeta, Sheno and Wolliso). After the routine laboratory analysis completed in the national HIV laboratory the leftover blood samples were used for evaluating the new single tube %$CD4^+$ T-cell determination system against that directly obtained from the FACSCalibur (TruCOUNT^{TM}). This new single tube FACSCount system was introduced by BD for field trial while this study was almost through.

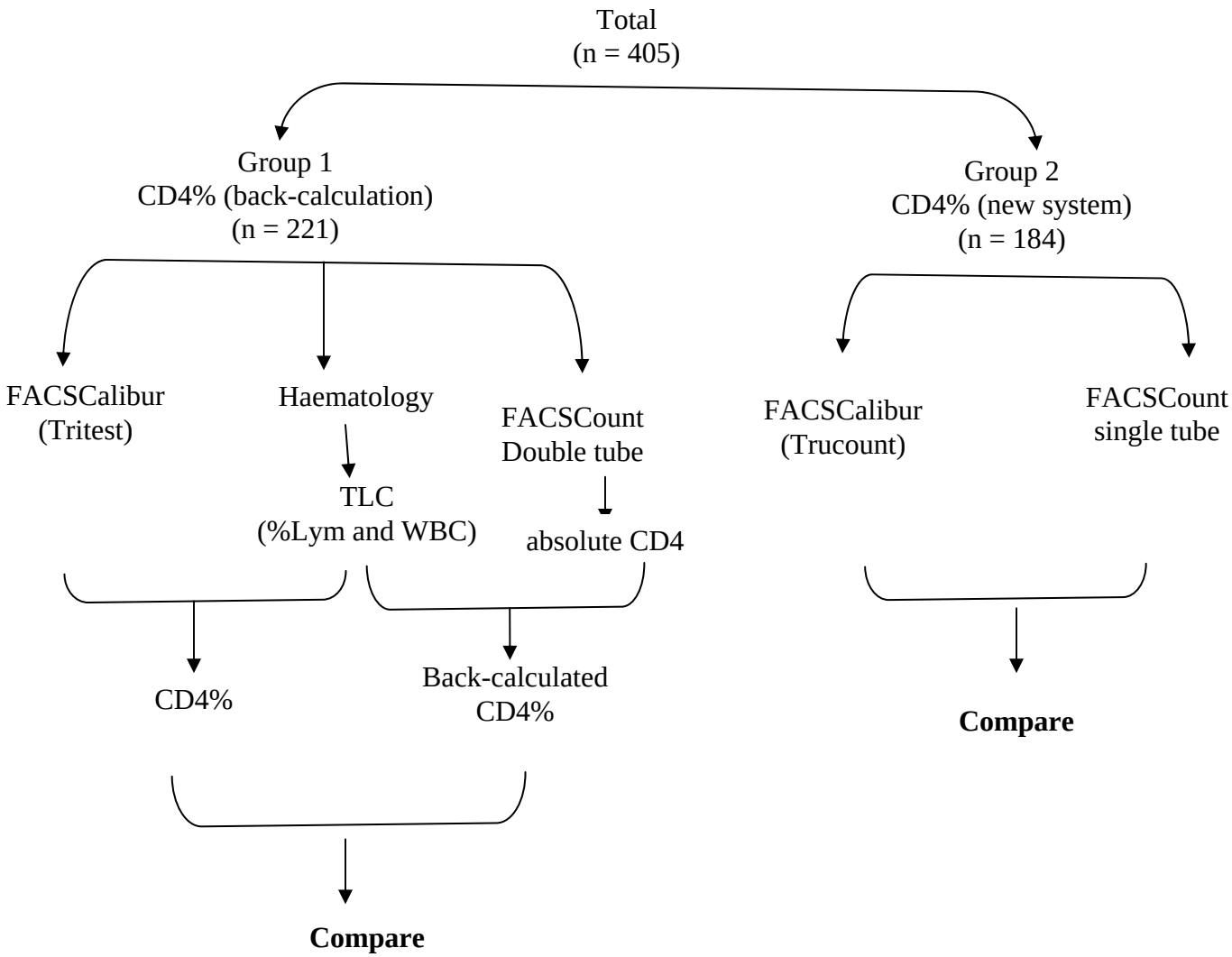
3.3. Inclusion criteria

HIV infected children below age 14 years attending ART clinics of BLH and ZMH (Group 1). Any sample referred to EHNRI national HIV laboratory (Group 2).

3.4. Sample size determination

This study used a sequential sampling approach by recruiting study participants as they present themselves for clinical care at the health care centers. A sample size of 40-100 is conventionally used for evaluation or method comparison and has been shown to assure a valid result, which either establish existence of no difference or confirms the existence of a difference (Linnet, 1999). Based on this a total of 405 samples were collected.

3.5 Work flow



3.5. Laboratory analysis

3.5.1. Samples, sample handling and preparation

A minimum of 2 ml leftover whole blood samples were obtained for each child included in the study from blood collected using K_3EDTA as part of routine clinical evaluations in the ART clinics. Each sample is collected in the morning at 10:00am. Each sample labeled appropriately and was prepared according to the standard operating procedure of the Ethiopian Health and Nutrition Research Institute national HIV laboratory. All the ART laboratories in and surrounding Addis Ababa were trained for appropriate sample handling and have the copy of the standard operating procedure. The tests were conducted at the national HIV laboratory of the Ethiopian Health and Nutrition Research Institute.

Hematological analysis

Automated haematological analysis was performed using Cell-Dyn 3700 (Abbot Diagnostics, USA) in order to get the total white cell count and lymphocyte percentage to be used by the existing double tube FACSCount as well as by the FACSCalibur for %$CD4^+$ T-cell determination (dual platform). The machine aspirated 130 µl of blood into a chamber and diluted with a balanced isotonic saline solution. The diluted blood sample is split into two parts: one goes for RBC and platelet counting and the other goes for total WBC and differential counting. The total leucocyte and lymphocyte counts were reported in microlitre.

Flow cytometric analysis using the existing double tube FACSCount system

The FACSCount is a SP system product of Becton Dickinson Biosciences (BDB, San Jose, California). It is a bench top flow cytometer consisting of a green HeNe laser light. The FACSCount system requires a ready to use paired-reagent tubes provided by the company. The reagent tubes are dedicated for enumeration of absolute $CD4^+$, $CD8^+$ and $CD3^+$ T-lymphocyte count in whole blood using a no-lyse and no-wash method. The first tube determines absolute number of $CD3^+$ $CD4^+$ T-cells using a combination of 2-colour monoclonal antibodies anti-human CD3 conjugated to the tandem dye PE-CY5 and anti-human CD4 conjugated to PE. The second tube determines the absolute number of $CD3^+$

$CD8^+$ T-cells using a combination of anti-human CD8 antibody conjugated to PE and anti-human CD3 conjugated to PE-CY5. Both tubes, in addition to the monoclonal antibodies, contain a known number of fluorochrome-labeled reference beads. The principle of the SP FACSCount system is based on the ratio of the number of events counted versus the number of reference beads counted by the machine as indicated in the formula given below. In the formula, the number of events represents number of $CD4^+$ or $CD8^+$ T-cells counted, and numbers of reference beads are known values given by the company.

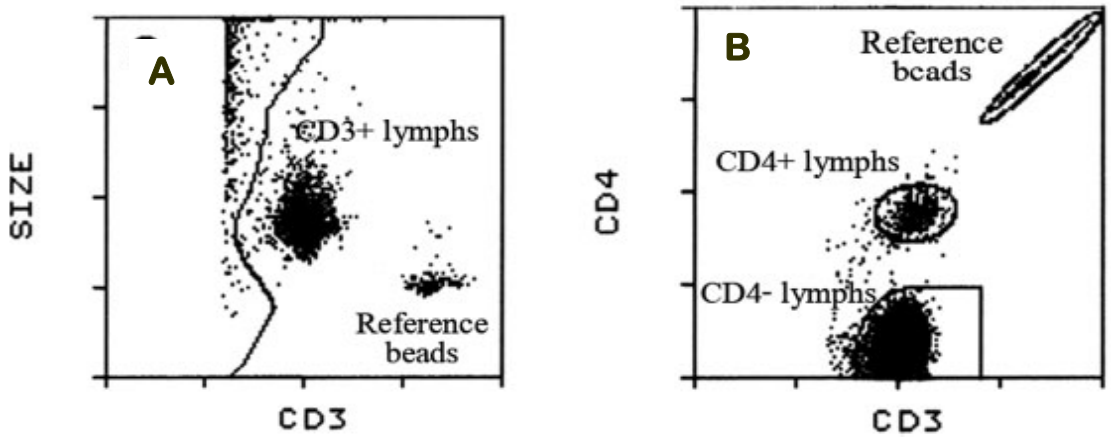


Figure 2. Dot plot software algorithm of the existing double tube two-colour FACSCount™.

$$\text{Abs. CD4 cells/}\mu L = \frac{\#\text{ events counted}}{\#\text{beads counted}} \times \frac{\#\text{ ref. beads}}{\text{Vol. blood}}$$

The procedure follows a whole blood no-lyse no-wash staining method. Briefly, exactly 50µl of whole blood from K_3EDTA collected tube was added using an automated calibrated electronic pipette to each tube provided by the company. The tubes were vortexed upside down then up right for 5 seconds and incubated for 60-120 minutes in the dark at room temperature. Then 50µl of fixative solution (5% formaldehyde in phosphate buffered saline) provided in the reagent kit was added into each tube. After the tubes were vortexed, stained samples were analyzed by the FACSCount double tube software. When a stained sample was introduced into the SP FACSCount system, $CD3^+$ T-cells and reference beads in the threshold gate were automatically generated by the built-in FACSCount software figure 2.

Elliptical regions and a quadrangular gate were automatically set around $CD3^+/CD4^+$ T-cells, $CD3^+/CD4^-$ T-cells and reference beads figure 2b. Sufficient numbers of cells (events up to 30,000 cells per sample) are collected. The ratio of fluorescent cells to beads multiplied by the known concentration of beads in the tube automatically give $CD3^+$, $CD4^+$ and $CD8^+$ T-lymphocyte counts. The absolute $CD4^+$ and $CD8^+$ T-cell counts are calculated as the absolute counts of $CD3^+$ T-lymphocytes only and not of acquired reference beads and $CD3^+$ T-lymphocyte gated in the threshold gate.

Flow cytometric analysis using the new single tube FACSCount system

The recently developed new FACSCount system requires single tube SP FACSCount CD4+ T-cell determination system. The new reagent system contains combination of a nuclear DNA fluorescent dye and three fluorescent conjugated monoclonal antibodies that identify CD4 positive lymphocytes and CD4 negative lymphocytes. The monoclonal antibodies are CD4-PE, CD14 PE-Cy5 and CD15 PE-Cy5, which are packed in the single test containing diluents and reference beads. Monoclonal antibody to CD14 recognizes a human monocyte/macrophage antigen, whereas monoclonal antibody to CD15 recognizes a human myelomonocytic antigen. The sample preparation and operation of the instrument is very similar to the procedure of the existing double tube FACSCount reagent but it takes a shorter staining period making it faster and more efficient.

In brief, 50µl of whole blood from K_3EDTA collected tube was added to the tube provided by the company using an electronic micropipette. The tube was vortexed upside down then up right for 6 seconds and incubated for 30-40 minute in the dark at room temperature. Then 50µl of fixative solution (5% formaldehyde in phosphate buffered saline) provided in the reagent kit was added to the tube. After the tube was vortexed, sample was analyzed by the FACSCount single tube software. When a stained sample was acquired total lymphocyte, $CD4^+$ T-lymphocyte and reference beads were identified by the in-built FACSCount software figure 3. Lymphocytes are identified by their DNA fluorescence and size while excluding nonlymphocytes from their CD14/CD15 expression (CD14 present on majority of peripheral

blood and CD15 present on more than 95% of mature peripheral blood eosinophils and neutrophils and at low density on circulating monocytes); consequently %$CD4^+$ T-cell was obtained automatically. The absolute number of $CD4^+$ T-lymphocyte was calculated by multiplying the ratio of fluorescent $CD4^+$ T-lymphocyte events by the known number of reference microbeads in tube.

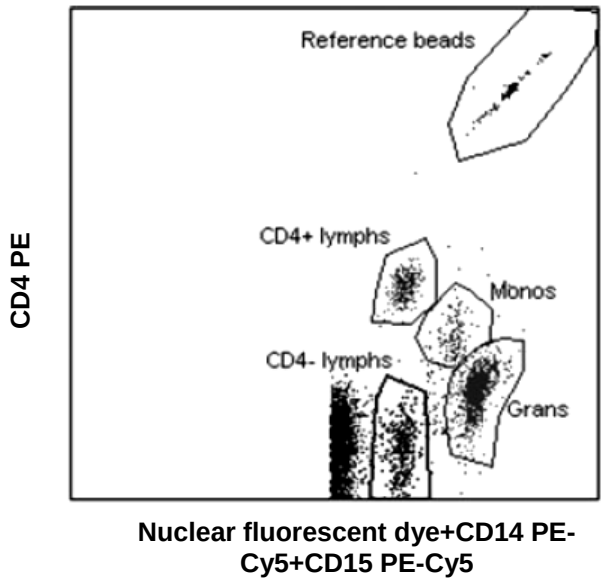


Figure 3. Dot plot software algorithm of the new single tube two-colour FACSCount^{TM}.

Flow cytometric analysis using FACSCalibur

FACSCalibur is the product of Becton Dickinson Biosciences (Beckton Dickinson, San Jose, California), with both DP and SP technology. A lyse/no-wash whole blood procedure was followed in this study. Both SP and DP FACSCalibur techniques were employed to ensure comparability with the SP and DP %$CD4^+$ T-cells obtained from the new and existing FACSCount systems, respectively. FACSCalibur TriTEST^{TM} system without introducing hematological (%lymphocyte and total WBC) results does not enables us to obtain absolute and %$CD4^+$ T-cells value which can be used for comparison with the back calculated %$CD4^+$ T-cells. FACSCalibur TruCOUNT ^{TM} system has a known number of reference beads to

obtain absolute and %$CD4^+$ T-cells directly without introducing hematological results which can be used for SP techniques.

$$\text{Abs. CD4 cell count} = \text{CD4\%} \times \text{TLC}$$

$$\text{CD4\%} = \frac{\text{Abs. CD4 count}}{\text{Lym\%} \times \text{tWBC}}$$

In brief, exactly 20µl of three-colour monoclonal antibodies (anti-human CD45 conjugated to the PerCP, anti-human CD3 conjugated to PE and anti-human CD4 conjugated to the FITC) and 50µl of K_3EDTA-anticoagulated whole blood obtained by reverse pipetting technique were mixed into TriTEST^{TM} (plain) and TruCOUNT ^{TM} tubes. The mixture was incubated for 15-20 minutes at room temperature in the dark place. Then, 450µl of 10% lysing solution which is prepared from the stock (100 ml of 10X buffered solution contains less than 50% diethylene glycol and less than15% formaldehyde) was added. After 15 minutes the sample was acquired and analyzed with FACSCalibur multiset software. When samples are stained with conjugated monoclonal antibodies (FITC, PE and PerCP) are detected using the logarithmic amplification of fluorescence 1 (FL1), FL2, and FL3 parameters. Forward scatter (FSC) and side scatter (SSC-H) signals were measured using a linear scale. After acquiring data on 15,000 cells, a region was automatically set on the lymphocyte cluster as R1 (SSC-H low/CD45 PerCP high1 cells). Cells outside this gate were considered as monocytes (SSC-H medium/CD45 PerCP intermediate1 cells) and granulocytes (SSC H high/CD45 low) figure 4a. Once the lymphocyte gate was established, percent double-positive $CD3^+/CD4^+$ T-lymphocyte in the upper right quadrant of a two-parameter dot-plot (Figure 4c) was then automatically generated. For absolute $CD4^+$ T-lymphocyte count, %$CD4^+$ T-cell is then multiplied by absolute lymphocyte count obtained from the hematology analyzer.

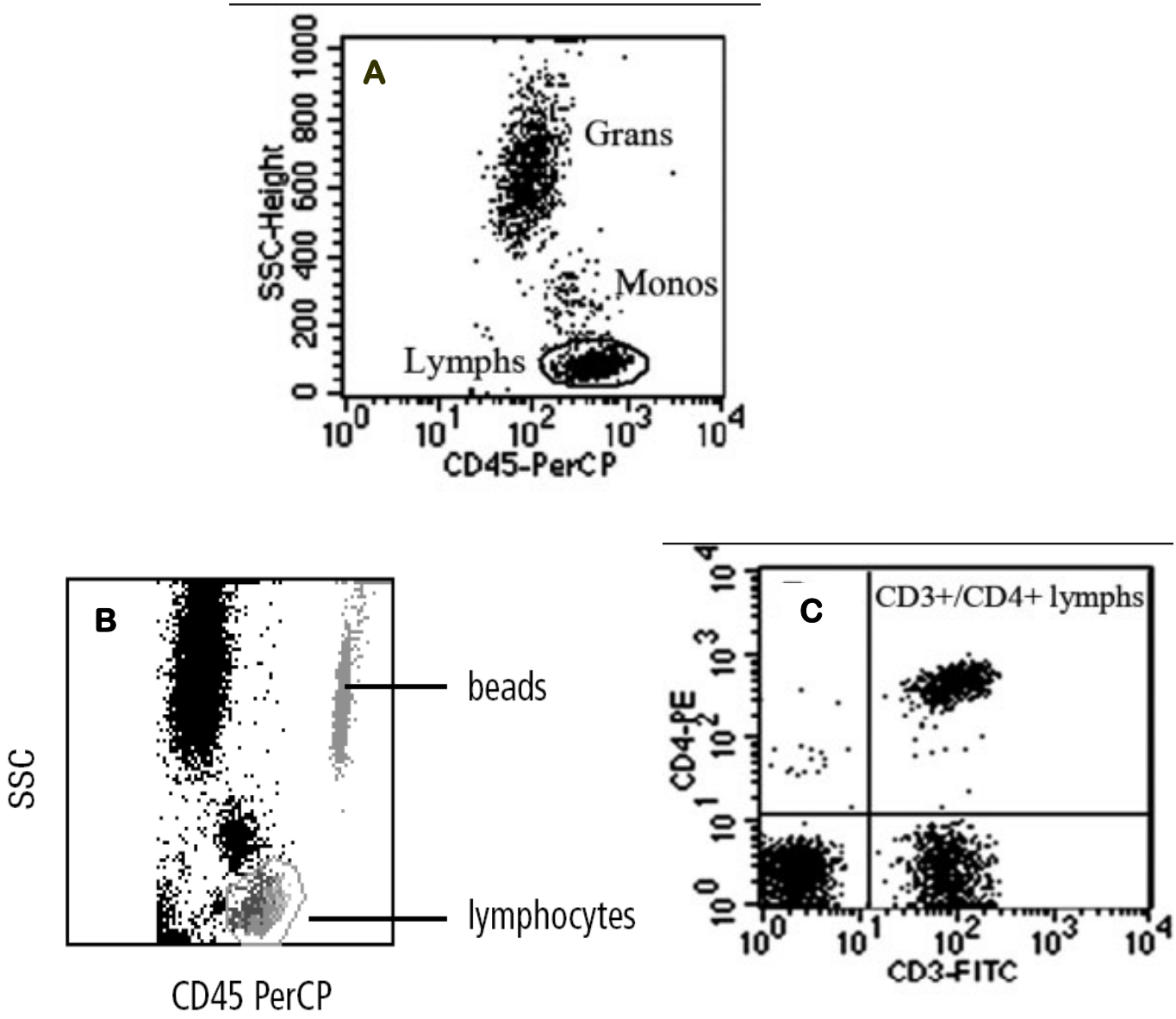


Figure 4. Dot plot software algorithm of three-color TriTEST™/MultiSET™ and TruCOUNT™/MultiSET™ DP and SP FSCACalibur™ system.

Quality control

When new technologies are integrated into diagnostic laboratory, the technologies should always undergo performance standard evaluation by following reagent and instrument (accuracy and precision) and quality control methods. Accuracy compares the test result to a reference or gold standard method whereas precision measures the reproducibility of a single

sample stained and analyzed in duplicate at least 8 times and coefficient of variation (CV) was set not more than 10% (Deems *et al.*, 2004).

To ensure quality control for the tests, test controls were run according to the daily start up procedures of each machine. For CELL-DYN®22, control is composed of stable materials that provide a means of verifying the accuracy and precision of Cell-Dyn hematology results. It was handled and run in the same manner as patient specimens. The control is available in 3 levels representing low, normal and high results. For FACSCount (double and single tube system) a control set of beads was used throughout the study. The control reagent consists of four (for double tube system) and three (for single tube system) known concentration of fluorescent beads. The zero beads (0 beads/µl), low (50 beads/µl), medium (250 beads/µl) and high (1000 beads/µl) were analyzed prior to measuring each batch of stained whole blood samples for the existing double tube SP FACSCount system. The low (50 beads/µl), medium (250 beads/µl) and high (1000 beads/µl) were analyzed prior to measuring each batch of stained whole blood samples for the new single tube SP FACSCount system as recommended by the company.

To ensure quality control of the SP and DP FACSCalibur results, as recommended by the company, photomultiplier tube voltage sensitivity and fluorescence compensation setting were optimized using control beads (CaliBRITE™) and FACSComp software weekly. In addition, the national HIV laboratory participates in external quality assurance programs for hematology and flow cytometry analyses to ensure the over all quality of the results.

3.6 Statistical analysis

Data were entered into excel sheet and analyzed using SPSS version 10.0 (Statistical Package for the Social Sciences Inc. 1999). Percentage $CD4^+$ T-cell obtained by back-calculation from the existing double tube FACSCount and from the new single tube FACSCount was correlated against FACSCalibur TriTEST™ and FACSCalibur TruCOUNT ™ systems using regression analysis.

Bland-Altman statistical bias plot is recommended to compare methods and to measure their limit of agreements (Bland and Altman, 1986) by analyzing using SigmaPlot Software version 11.0. It is employed by plotting the average of the values obtained from the two methods on the x-axis and the difference between the two methods on the y-axis. The bias (mean difference) and limit of agreement (LOA) [mean ± 2 standard deviation], confidence intervals (95%) for the mean difference and limit of agreement are calculated to determine the range on which the true values may lie. Two methods compare favorably when the mean difference is close to zero, limit of agreement are narrow and few outliers are present (Scott *et al.,* 2003). The statistical significance was considered when p-value was less than 0.05.

Percent similarity was calculated *[({a+b}/2)/a]x100*, where a and b are averages of the evaluated and the gold standard systems (Scott *et al.,* 2003). Data pairs with the same value will be 100% similar. If the new method is greater than the gold standard percent similarity will be greater than 100% and if the new method is less than the gold standard percent similarity will be less than 100%.

3.7 Ethical Considerations

Blood samples were collected for routine pediatric HIV management in an unlinked and anonymous manner. Coded leftover samples were used from the routine ART laboratory analysis. Ethical clearance was obtained from the ethical review committees of Addis Ababa University, Science Faculty, Department of Biology and Medical Faculty, Department of Pediatrics and from Ethiopian Health and Nutrition Research Institute (EHNRI).

4. Result

4.1. Method precision

This study has to pass precision test for comparing two methods, which was set as a coefficient of variation (CV) not more than 10%. As shown in table 2, all systems showed good precision in both methods (single and dual platform) and instruments (FACSCount^{TM} and FACSCalibur^{TM}) having CV less than 8.5% for 8 duplicate processes by the two methods. The determination of absolute $CD4^+$ T-cell obtained the existing double tube SP FACSCount, the new single tube SP FACSCount system and the SP and DP FACSCalibur from 8 separated processes showed CV 3.1%, 5.2%, 4.9% and 8.4%, respectively. For the determination of $\%CD4^+$ T-cell count obtained the existing double tube FACSCount, the new single tube SP FACSCount system and the SP and DP FACSCalibur from 8 separated processes showed CV 7.1%, 2.1%, 2% and 3.2%, respectively.

Table 2. Instrument precision test for (1) the existing double tube FACSCount system (2) FACSCalibur TriTEST^{TM} system (3) the new single tube FACSCount and (4) FACSCalibur TruCOUNT system.

No	WBC	Lymphocyte	TLC	Existing double tube FACSCount		FACSCalibur TriTEST™			New single tube FACSCount		FACSCalibur TruCOUNT™	
				absolute CD4	Back-calculated %CD4	absolute CD4	%CD4		Absolute CD4	%CD4	absolute CD4	%CD4
1	4600	40	1840.0	748	41	707	38		730	40	734	35
2	4680	39	1834.6	731	40	727	39		814	41	635	37
3	4520	38	1713.1	740	43	680	40		806	40	832	35
4	4510	39	1740.9	729	42	689	40		786	41	783	35
5	4840	39	1882.8	730	39	740	40		763	41	684	38
6	4890	41	2009.8	696	35	789	39		735	39	786	37
7	4640	38	1777.1	776	44	686	39		710	39	771	37
8	4670	39	1812.0	726	40	688	38		747	41	759	36
Mean	4668.8	39.1	1826.3	734.5	40.3	713.3	39.1		761.4	40.1	748.0	36.3
SDv	137.0	1.0	92.3	22.5	2.9	37.3	0.8		37.6	0.8	62.6	1.2
C.V.	2.9	2.6	5.1	3.1	7.1	5.2	2.1		4.9	2.0	8.4	3.2

4.2. Demographic and clinical characteristics of the study group

During the study period a total of 405 HIV infected patients were enrolled. For the evaluation of comparison of the back calculated against the FACSCalibur generated %$CD4^+$ T-cells , a total of 221 children below 15 years of age were enrolled (Group 1). These children were attending pediatric ART centers of Black Lion Teaching Generalized Referral Hospital (BLH, n=169) and Zewditu Memorial Hospital (ZMH, n=52). In addition, referral whole blood samples from 184 subjects composed of children and adults aged 1.2 to 66 years (Group 2) were utilized for the evaluation of %$CD4^+$ T-cells generated from the recently introduced single tube single platform new reagent FACSCount system versus %$CD4^+$ T-cells from the FACSCalibur. The blood samples were referred to the Ethiopian Health and Nutrition Research Institute national HIV laboratory, following standard sample referral procedures, from ART centers around Addis Ababa, which had no or non functional FACSCount machine.

Demographic and clinical data for Group 1 study subjects, including WHO staging and ART status, were collected from the log books at the ART centers. For Group 2, data were collected from the referral requisition form. Table 3 shows the demographic and clinical data of children attending pediatric ART clinics of ZMH and BLH. Information on age, gender and WHO staging could not be obtained for 50 children (22.6%), since their medical records were transferred to their nearest health facility. As a result mean and median age of the study subjects were not analyzed. The majority of the remaining children belong to the age group 5 to 14.9 years (50.7%) followed by 3 to 4.9 years (19%). As shown in table 3, additional information on ART status was obtained for 164 pediatric patients in Group 1. Accordingly, 60.2 % (133/164) were ART experienced and 14% (31/164) were ART naïve while for the remaining 57 children ART status data could not be obtained since records were transferred to their nearest health facility for follow up.

Table 3. Demographic and clinical data of pediatric patients attending ZMH and BLH ART centers (Group 1), Addis Ababa, 2008 (N=221).

Demographic characteristics of Group 1		**N (%)**
Age		
	<1 year	1 (0.5)
	1-2.9	16 (7.2)
	3-4.9	42 (19)
	5-14.9	112 (50.7)
	Unknown	50 (22.6)
Gender		
	Female	72 (32.6)
	Male	99 (44.8)
	Unknown	50 (22.6)
WHO Staging		
	I (Asymptomatic)	12 (5.4)
	II (Mild)	50 (22.6)
	III (Advanced)	67 (30.3)
	IV (Severe)	42 (19)
Treatment		
	ART experienced	133 (60.2%)
	Naïve	31 (14%)
	Unknown	57 (25.8%)

Table 4. Demographic and clinical data of patients referred to EHNRI (Group 2), Addis Ababa, 2008 (N=184).

Demographic characteristic of Group 2		**N (%)**
Age		
	<15	21 (100)
Gender		
	Female	12(57.14)
	Male	9(42.86)
WHO Staging		
	I (Asymptomatic)	6 (28.57)
	II (Mild)	5 (23.81)
	III (Advanced)	6 (28.57)
	IV (Severe)	1 (4.76)
	Unknown	3 (14.29)

Demographic and clinical data for Group 2 (n=21) subjects whose whole blood samples referred to EHNRI for $CD4^+$ T cell counting is shown in Table 4. There were more female children (57.14%) than male children (42.86%). WHO staging data was unavailable in the referral requisition form for three study subjects. The remaining subjects were in WHO stage I (28.57%), II (23.81%), III (28.57%) followed by Stage IV (4.76%).

Additionally information for Group 1 regarding hematology, absolute $CD3^+$ and $CD4^+$, and back-calculated $\%CD4^+$ T-cells were collected after the tests were performed at EHNRI national HIV laboratory. The mean, median and interquartile range (IQR) of TLC, WBC, lymphocyte and hemoglobin are presented in table 5. The overall median of TLC, WBC, lymphocyte and hemoglobin are 2.6 cells/µl (Interquartile range (IQR:1.8-3.2), 5.9 cells/µl (IQR:4.5-7.7), 45 cells/µl (IQR:36-57) and 12.9 mg/dl (IQR:12.1-13.6) respectively.

Table 5. Hematological profile of pediatric patients attending ZMH and BLH ART centers, Addis Ababa. (N=221)

Hematological profile	**Mean ±SD**	**Median and IQR**
TLC ($x10^9$/l)	2.7 ± 1.5	2.6 (1.8-3.2)
WBC ($x10^9$/l)	6.2 ± 2.5	5.9 (4.5-7.7)
Lymphocyte (%)	46.0 ± 14.5	45 (36-57)
Hemoglobin (gm/dl)	12.8 ± 1.5	12.9 (12.1-13.6)

4.3 Evaluation of back-calculated versus FACSCalibur generated %CD4+ T-cells

Absolute $CD3^+$, $CD4^+$ and back-calculated $\%CD4^+$ T-cells were determined using the existing double tube FACSCount system and FACSCalibur (plain tube TriTEST^{TM}) followed the dual platform technology. The total number of patients whose absolute $CD3^+$, $CD4^+$ and back-calculated $\%CD4^+$ T-cell values analyzed by the two systems was 221 and the mean, median and interquartile range of hematalogical parameters were determined and presented in table 6. The means of (Group-1) absolute $CD3^+$ and $CD4^+$ T-cells using existing double

tube FACSCount system and FACSCalibur (plain tube TriTEST^{TM}) are 2139.7 cells/µl and 617.8 cells/µl respectively. The median of absolute CD3^{+} T-cell count from the existing double tube FACSCount is 2099 cells/µl (IQR: 1534-2686) while from that of FACSCalibur TriTEST^{TM} is 2064 cells/µl (IQR: 1444-2592). The median of absolute CD4^{+} T-cell count from the existing double tube FACSCount is 564 cells/µl (IQR: 352-852) while from that of FACSCalibur TriTEST^{TM} is 537 cells/µl (IQR: 330-824.). The means of back-calculated %CD4+ T-cell from the existing double tube FACSCount and FACSCalibur TriTEST^{TM} are 22.2% and 23.3% and the median are 22% (IQR: 16-28) and 22.6% (IQR: 16.1-30.4). Both mean and median value of %CD4+ T-cells from back-calculation and FACSCalibur are similar but not statistically significant (p> 0.05).

Table 6. The mean and median and interquartile range of absolute CD3^{+}, CD4^{+} and %CD4^{+} T-cells of pediatric patients attending ZMH and BLH ART centers using the two systems (N=221).

	Mean ± SD		**Median and IQR**	
	Existing double tube FACSCount	**FACSCalibur (plain tube TriTEST^{TM})**	**Existing double tube FACSCount**	**FACSCalibur (plain tube TriTEST^{TM})**
CD3+ T-cells	2139.7 ± 797.3	2146.7 ± 956.9	2099 (1534-2686)	2064 (1444-2592)
Absolute CD4+ T-cells	617.8 ± 338.7	588.1 ± 333.7	564 (352-852)	537 (330-824)
%CD4+ T-cell	22.2 ± 9.4*	23.3 ± 10.6	22 (16-28)*	22.6 (16.1-30.4)

* - back-calculated %CD4+ T-cells

Correlation linear regression analysis for back-calculated %CD4^{+} T-cells obtained from the existing double tube FACSCount against DP FACSCalibur TriTEST^{TM} were shown in table 6 and figure 5. Percentage CD4^{+} T-cells obtained by back-calculation from the existing double tube FACSCount and directly from DP FACSCalibur TriTEST^{TM} has shown a good correlation ($r = 0.960$, $y = 1.0294x + 0.3208$; $p<0.0001$). The Bland-Altman plot analysis showed an overall bias of +1.6% (with LOA from –2.3% to +4.2%). At the same time percent similarity for the back-calculated %CD4^{+} T-cells obtained from the existing double tube FACACount system was analyzed against %CD4^{+} T-cells obtained from the DP

FACSCalibur TriTEST™, and percent similarity was 102.2% (CV = 7.8) is observed indicating that both systems show good agreement.

Table 7. Correlation coefficient analysis of %$CD4^+$ T-cells and using the double tube FACSCount and FACSCalibur TriTEST™ system based on the WHO immunological criteria of pediatrics.

The existing double tube FACSCount system vs FACSCalibur (plain tube TriTEST™)		Mean Diff. (%)	LOA (%)	R	Mean similarity (%)	CV	P-value
Absolute CD4+ T-cell count		29.7	-69.6 to +128.9	0.954	97.6	8.2	0.0001
	<200 cells/mm^3	-7.8	-48 to +32.3	0.765	95.8	16.3	0.0001
	200-350 cells/mm^3	14.6	-34.5 to +63.8	0.755	98.2	9	0.0001
	350-750 cells/mm^3	24.2	-58.4 to +111.6	0.903	97.5	8.2	0.0001
	>750 cells/mm^3	36.5	-104.2 to +203.6	0.902	99.5	7.9	0.0001
Back-calculated %CD4+ T-cell		1.6	-2.3 to +4.2	0.960	102.2	7.8	0.0001
	<15%	-0.7	-1.5 to +4.5	0.902	103.6	9.7	0.0001
	15%-24%	-0.7	-2.5 to +6.3	0.875	101.7	6	0.0001
	≥25%	-0.9	-3.5 to +6.4	0.901	101.5	7.7	0.0001

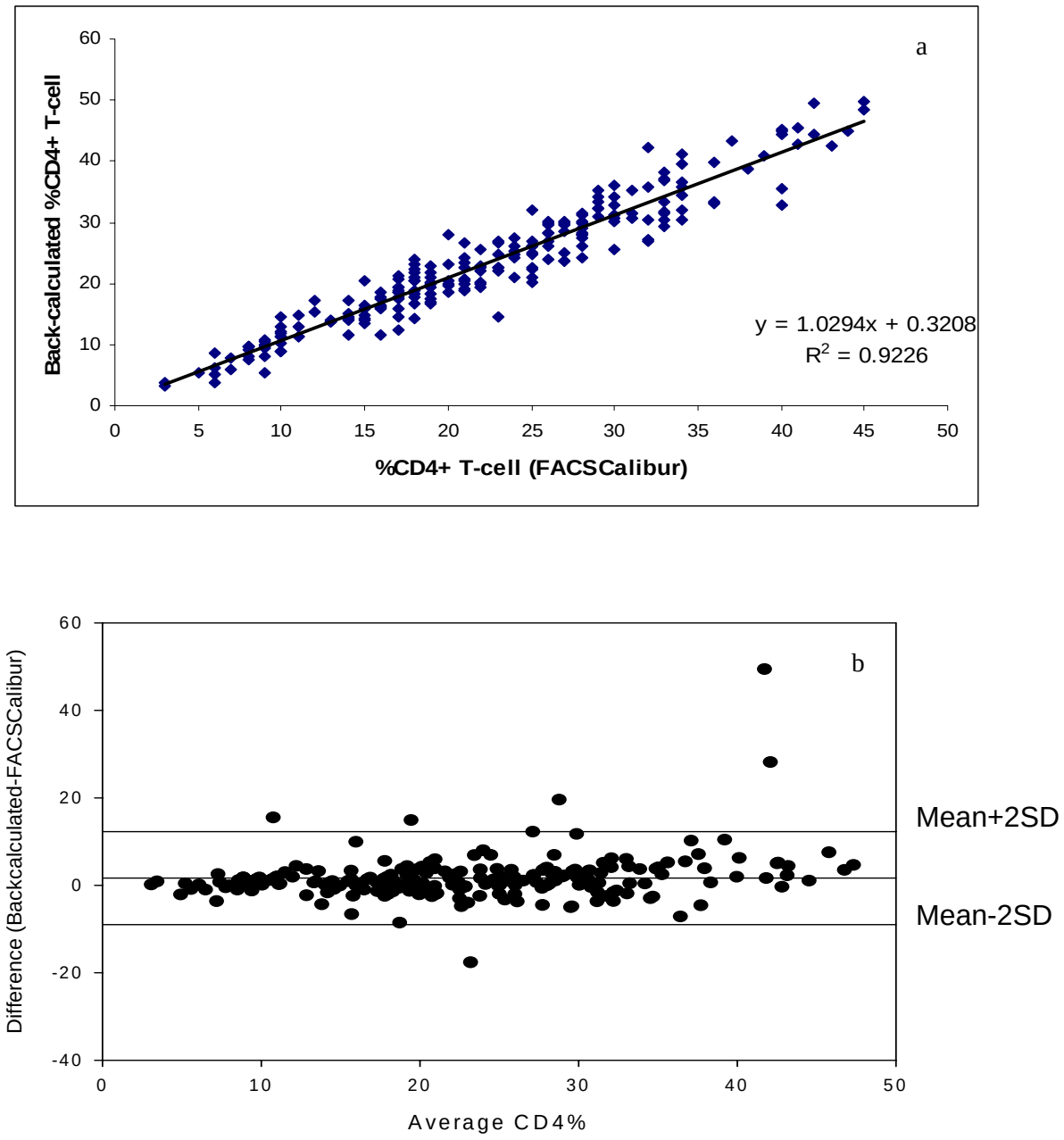


Figure 5. A correlation linear regression (a) and Bland-Altman bias plot analysis (b) of %CD4$^+$ T-cells values determined for group 1 study population obtained from the existing double tube FACScount system and DP FACSCalibur TriTEST^{TM} system.

Similarly correlation linear regression and Bland-Altman plot analysis of absolute CD4$^+$ T-cells obtained from the existing double tube FACSCount and directly from DP FACSCalibur were shown on table 6 and figure 6. Absolute CD4$^+$ T-cells obtained from the existing double

tube FACSCount system and the DP FACSCalibur has shown in general good correlation (r = 0.954, y = 0.9096x + 48.456, p<0.0001). The Bland-Altman plot analysis showed an overall bias of +29.7cells/µl (with LOA from –69.6 to +128.9). When the existing double tube FACSCount system was compared to the DP FACSCalibur a similarity of 97.6% with CV 8.2% was observed.

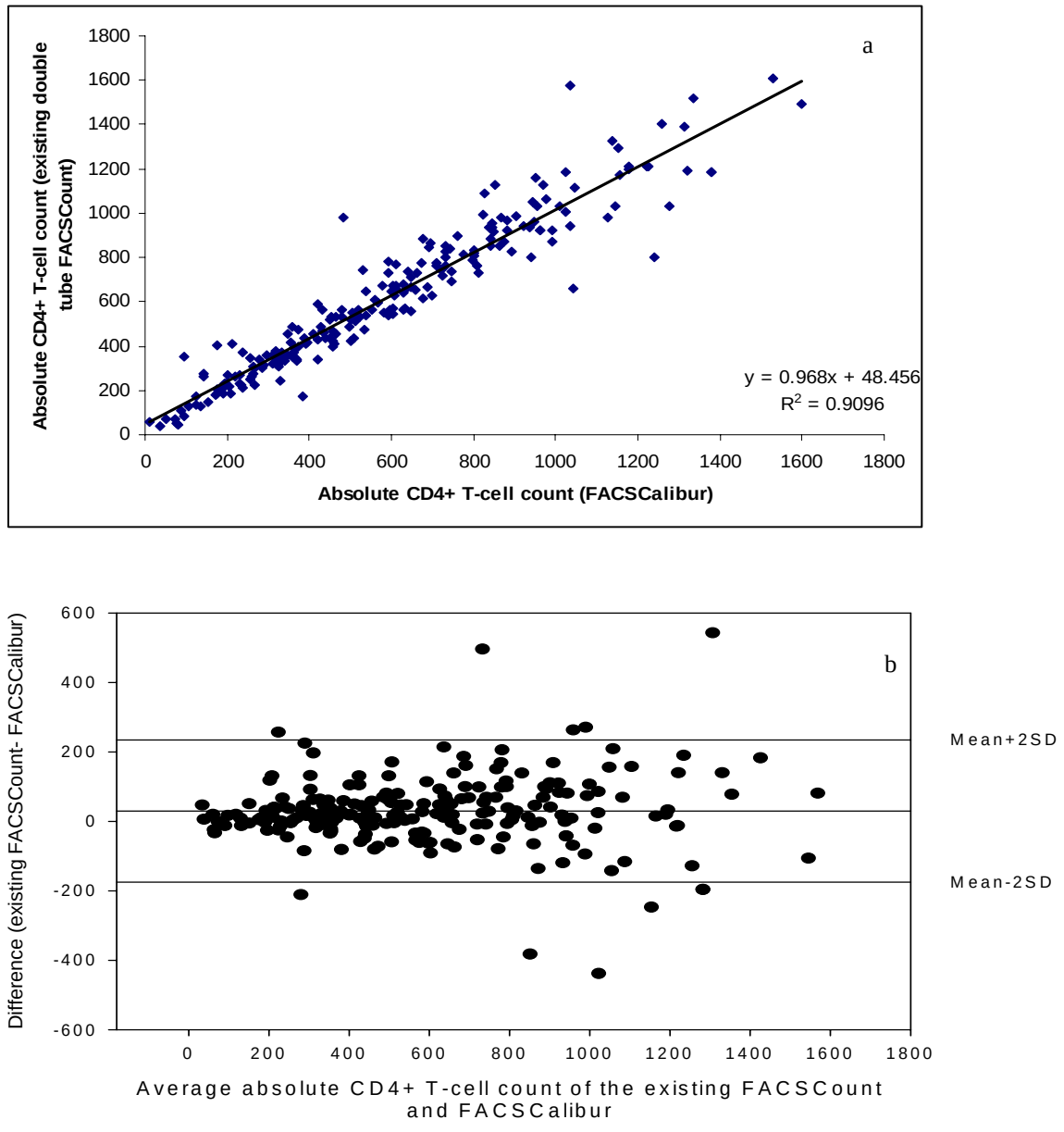


Figure 6. A correlation linear regression analysis (a) and Bland-Altman bias plot (b) of absolute $CD4^+$ T-cell count determined for group 1 study population by the existing double tube FACScount system and DP FACSCalibur TriTEST™ system.

Also correlation linear regression and Bland-Altman plot analysis was performed based on WHO immunological criteria for pediatrics. Even though the two methods are significantly and positively correlated still a lower correlation has been observed in children whose absolute $CD4^+$ T-cell counts are below 350 cells/mm3 ($r = 0.765$, $p<0.0001$; $r = 0.755$, $p<0.0001$) and $\%CD4^+$ T-cell in between 15-24% ($r = 0.875$, $p<0.0001$). During the time more than 95% percent similarity and a CV below 10% were observed for all category except that in children whose absolute $CD4^+$ T-cell count below 200 cells/mm3 and a CV of 16.3% indicating results from the two systems vary more than 10%.

4.4 Evaluation of %CD4+ T-cells generated from the new single tube FACSCount and FACSCalibur system

Absolute $CD4^+$ and $\%CD4^+$ T-cells were determined by using the new single tube FACSCount system and FACSCalibur TruCOUNT™ followed the single platform technology. The total number of patients whose $CD4^+$ and $\%CD4^+$ T-cell values analyzed by the two systems was 21. The mean, median and interquartile range of absolute $CD4^+$ and $\%CD4^+$ T-cells by the two systems were determined accordingly and presented in table 7.

Table 8. The mean ±SD and median and interquartile range of absolute CD4+ and percent CD4 T-cells

Below 15		Mean ± SD		Median and IQR	
		The new single tube FACSCount system	FACSCalibur (TruCOUNT™) system	The new single tube FACSCount system	FACSCalibur (TruCOUNT™) system
	Absolute $CD4^+$ T-cells	792.8±513.1	771.8±460.9	587 (552-929)	676 (559-785)
	$\%CD4^+$ T-cells	22.5±9.2	24.2±9.3	20 (17.85-30.8)	23(19-34)

The mean absolute $CD4^+$ T-cells of children by the two systems are 792.8 cells/µl and 771.8 cells/µl. The mean $\%CD4^+$ T-cells of children by the two systems are 22.5% and 24.2%, respectively. The $\%CD4^+$ T-cells from new single tube FACSCount system and FACSCalibur TruCOUNT™ systems show a very close mean of 22.5% and 24.2%. The median and interquartile range of absolute $CD4^+$ and $\%CD4^+$ T-cells are also given in table

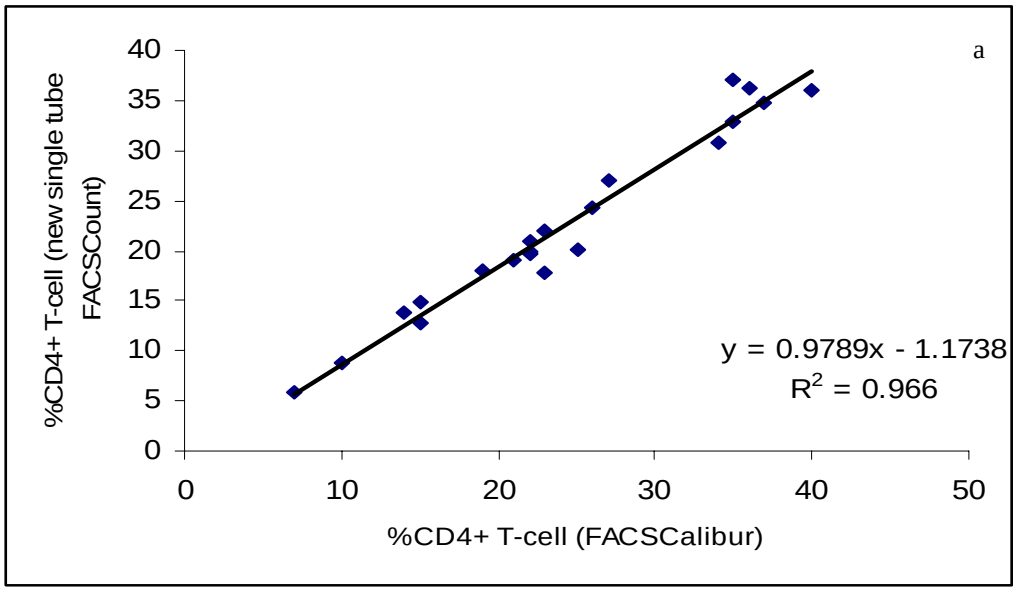
8. The overall median of %CD4$^+$ T-cell of children using the two systems 20 cells/μl (IQR: 17.85-30.77) and 23 cells/μl (IQR: 19-34), respectively.

Table 9. Correlation coefficient analysis of CD4$^+$ and %CD4$^+$ T-cells using the new single tube FACSCount system versus FACSCalibur (TruCOUNT^{TM}) system based on their age category.

The new single tube FACSCount system vs FACSCalibur (TruCOUNT™) system		Mean Diff.	LOA (%)	r	Mean similarity (%)	CV	P-value
Below 15	Absolute CD4$^+$ T-cells	21	-92.5 to +134.4	0.976	101.4	7.2	0.0001
	%CD4$^+$ T-cells	1.7	-5.2 to +1.5	0.983	96.5	5.7	0.0001

Correlation linear regression and Bland-Altman plot analysis for %CD4$^+$ T-cells obtained from the new single tube SP FACSCount against the SP FACSCalibur TruCOUNT^{TM} were shown in table 9 and figure 7. Percentage CD4+ T-cells obtained from the single tube SP FACSCount and from the FACSCalibur TruCOUNT^{TM} directly has shown a good correlation ($r = 0.983$, $y = 0.9789x - 1.1738$; $p<0.0001$) (Figure 7a and b) with an overall bias +1.7% (with LOA from –5.2% to +1.5%). The percent similarity of %CD4$^+$ T-cells is 96.5% having the coefficient of variation of 5.7%.

At the same time correlation linear regression and Bland-Altman plot analysis of absolute CD4$^+$ T-cells obtained from the new single tube SP FACSCount and from SP FACSCalibur TruCOUNT^{TM} shown in table 9 and figure 8. Absolute CD4+ T-cells obtained from the new single tube SP FACSCount system and the SP FACSCalibur TruCOUNT^{TM} has shown good correlation ($r = 0.9761$, $y = 1.0868x - 46.018$) with an overall bias +21% (with LOA from –92.5% to +134.4%). The percent similarity of absolute CD4$^+$ T-cells was 101.4% having the CV 7.2%. When the two systems were compared they have shown good percent similarity and CV below 8.5%.



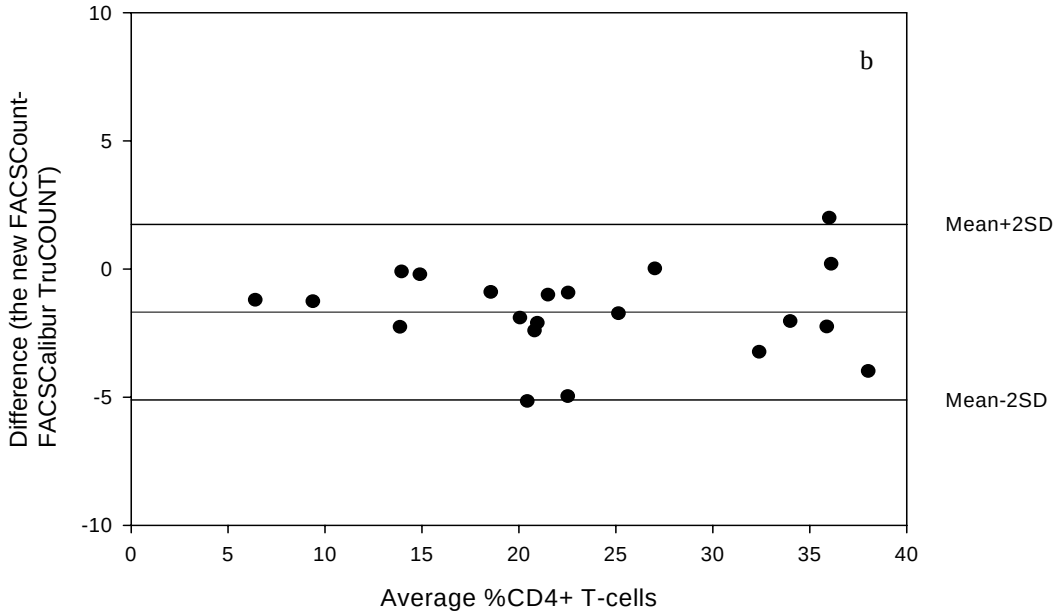
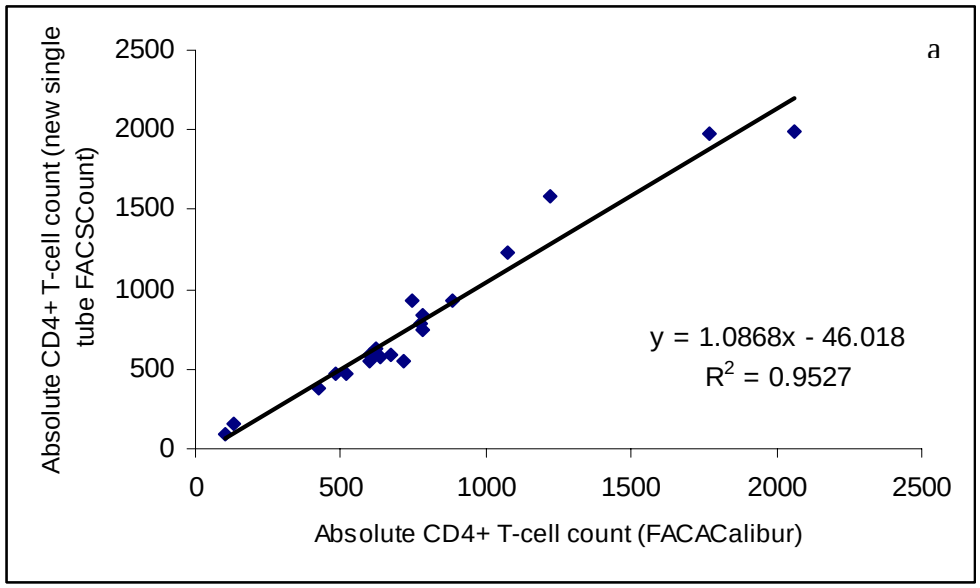


Figure 7. A correlation linear regression and Bland-Altman bias plot analysis of %CD4+ T-cells count determined for group 2 study populations by the new single tube SP FACSCount system and SP FACSCalibur TruCOUNT system. Linear regression analysis of %CD4+ T-cells (a) and Bland-Altman plot analysis of %CD4+ T-cells of children below 15 years (b).



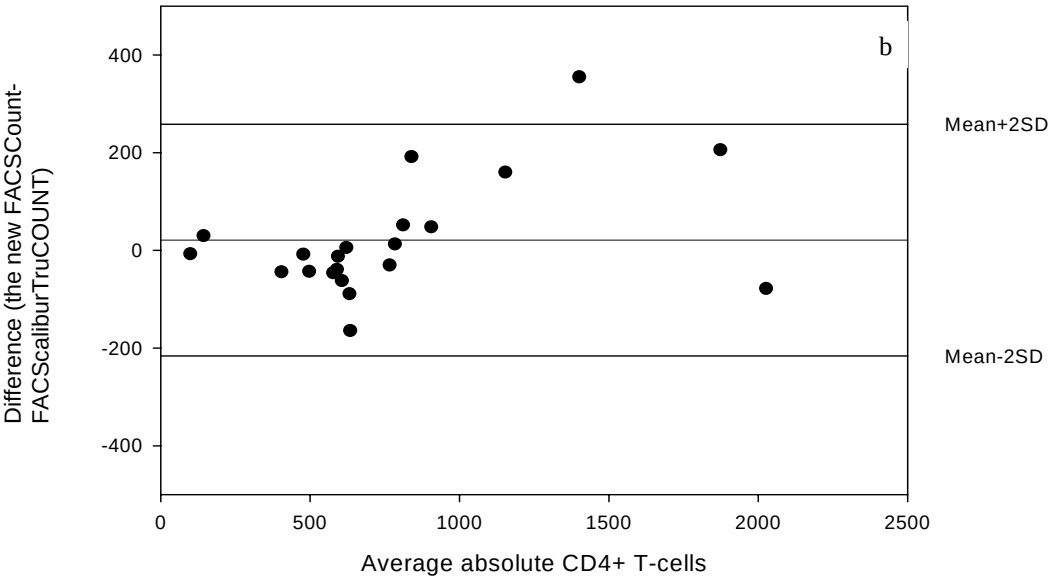


Figure 8. A correlation linear regression and Bland-Altman bias plot analysis of absoluteCD4+ T-cells count determined for group 2 study populations by the new single tube SP FACSCount system and SP FACSCalibur TruCOUNT system. Linear regression analysis of absolute CD4+ T-cells (a) and Bland-Altman plot analysis of absolute CD4+ T-cells of children below 15 years (b).

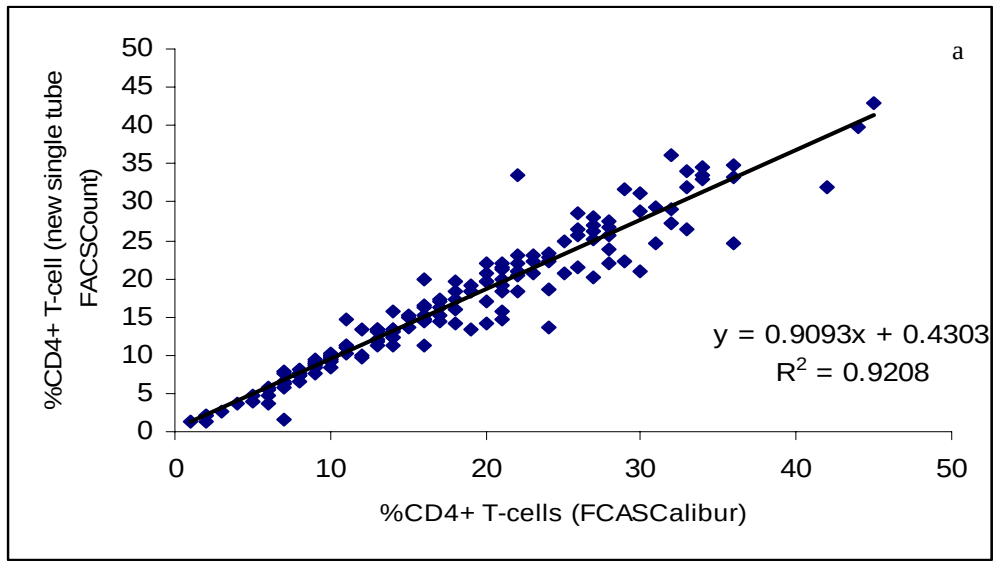
4.4.1 Evaluation of %CD4+ T-cells generated from the new single tube FACSCount and FACSCalibur system for adults

The total number 163 adult patients absolute CD4+ T-cell count and %CD4+ t-cell counts were correlated using the new single tube SP FACSCount against the SP FACSCalibur TruCOUNT^{TM}. Correlation linear regression and Bland-Altman plot analysis for %CD4^{+} T-cells obtained from the new single tube SP FACSCount against the SP FACSCalibur TruCOUNT^{TM} were shown in table 10 and figure 9. Percentage CD4+ T-cells obtained from the single tube SP FACSCount and from the FACSCalibur TruCOUNT^{TM} directly has shown a good correlation ($r = 0.960$, $y = 0.9093x\ 0.4303$; $p<0.0001$) with an overall bias -1.2% (with LOA from –3.7% to +1.4%). The percent similarity of %CD4^{+} T-cells is 96.9% having the coefficient of variation of 7.4%.

Table 10. Correlation coefficient analysis of CD4^{+} and %CD4^{+} T-cells using the new single tube FACSCount system versus FACSCalibur (TruCOUNT^{TM}) system for adults.

The new single tube FACSCount system vs FACSCalibur (TruCOUNT™) system		Mean Diff.	LOA (%)	r	Mean similarity (%)	CV	P-value
Above or equal to15	Absolute CD4^{+} T-cells	-10.0	-65.3 to +45.3	0.970	98.5	8.2	0.0001
	%CD4^{+} T-cells	-1.2	-3.7 to +1.4	0.960	96.9	7.4	0.0001

At the same time correlation linear regression and Bland-Altman plot analysis of absolute CD4^{+} T-cells obtained from the new single tube SP FACSCount and from SP FACSCalibur TruCOUNT^{TM} shown in table 10 and figure 10. Absolute CD4+ T-cells obtained from the new single tube SP FACSCount system and the SP FACSCalibur TruCOUNT^{TM} has shown good correlation ($r = 0.970$, $y = 0.903x + 23.161$) with an overall bias -10% (with LOA from –65.3% to +45.3%). The percent similarity of absolute CD4^{+} T-cells was 98.5% having the CV 8.2%. When the two systems were compared they have shown good percent similarity and CV below 8.5%.



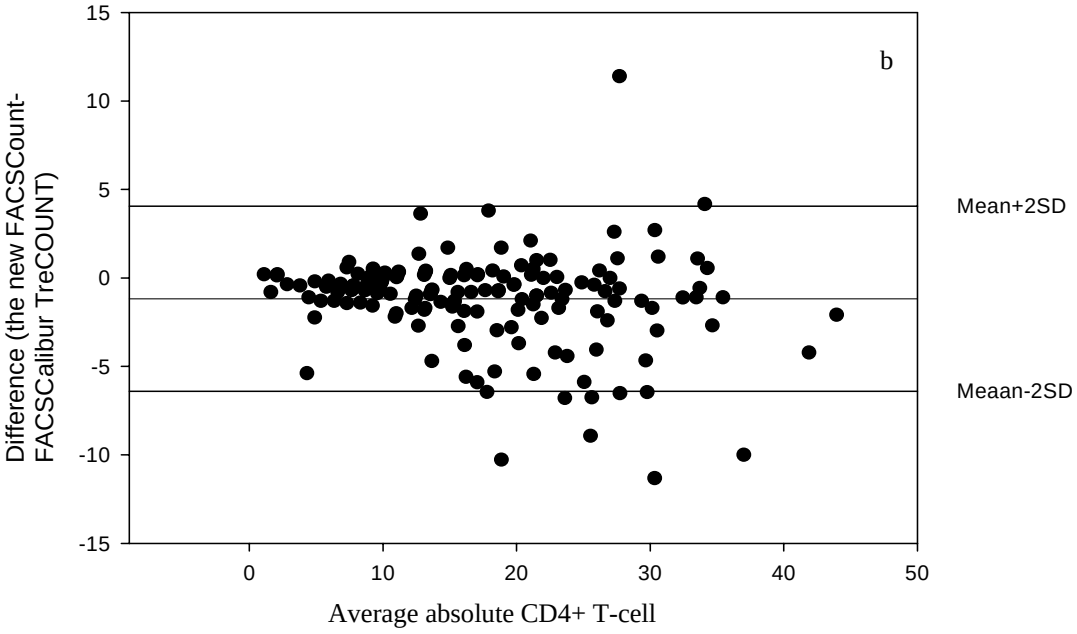


Figure 9. A correlation linear regression and Bland-Altman bias plot analysis of absoluteCD4+ T-cells count determined for group 2 study populations by the new single tube SP FACSCount system and SP FACSCalibur TruCOUNT system. Linear regression analysis of absolute CD4+ T-cells (a) and Bland-Altman plot analysis of absolute CD4+ T-cells of adults above 15 years (b).

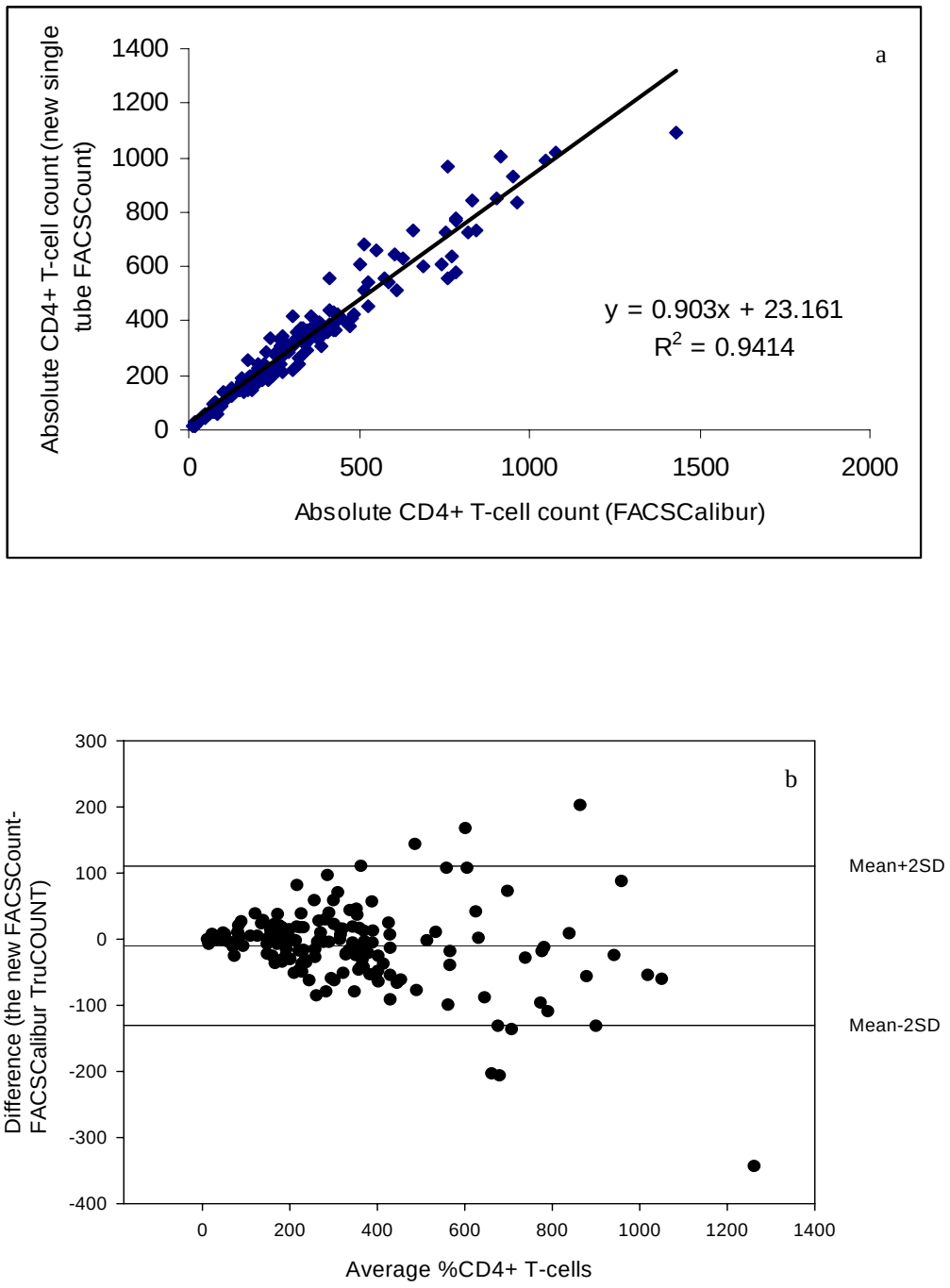


Figure 10. A correlation linear regression and Bland-Altman bias plot analysis of %CD4+ T-cells count determined for group 2 study populations by the new single tube SP FACSCount system and SP FACSCalibur TruCOUNT system. Linear regression analysis of %CD4+ T-cells (a) and Bland-Altman plot analysis of %CD4+ T-cells of adults above 15 years (b).

5 Discussion

Monitoring of the immune status of HIV-infected children through $CD4^+$ T-cell count requires accurate and precise methods for obtaining results that help in managing children before and after initiation of ART. In the current practice of HIV monitoring and care, there are continuous operational studies and several of them are involved with method validation (Pattanapanyasat *et al.,* 2005; Zijenah *et al.,* 2006; Manasa *et al.,* 2007; Jeganathan *et al.,* 2008; Pattanapanyasat *et al.,* 2008). The intention of this study was evaluation of the back-calculated $\%CD4^+$ T-cells obtained from the existing double tube SP FACSCount against the DP FACSCalibur TriTEST™ system and evaluation of the newly introduced single tube, SP FACSCount system and the SP FACSCalibur TruCOUNT™ system.

In this study FACSCalibur (TriTEST™ and TruCOUNT™) system is the gold standard and the single and double tube FACSCount systems are being evaluated against the $\%CD4^+$ T-cell values. The DP FACSCalibur TriTEST™ has produced more precise and reproducible $\%CD4^+$ T-cell values (CV 2.1%) than that of the back calculated $\%CD4^+$ T-cell values (CV 7.1%). Similarly the new single tube SP FACSCount systems have produced more precise and reproducible $\%CD4^+$ T-cell values (CV 2%) than that of the SP FACSCalibur TruCOUNT™ $\%CD4^+$ T-cell values (CV 3.2%). Coefficient of variation of absolute CD4+ T-cell count observed from the double tube and single tube FACSCount are 3.1% and 4.9% whereas the CV of absolute CD4+ T-cell count from the SP and DP FACSCalibur are a bit higher (5.2% and 8.4%) which might be due to manual gating. A higher CV seen in back calculated $\%CD4^+$ T-cell might be due to variable results which are most likely from hematological analyzer (Malone *et al.,* 1991).

In pediatric patients $\%CD4^+$ T-cell value is a more useful parameter for monitoring HIV infection as it varies less significantly than the absolute CD4+ T-cell count. Thus it is of practical importance that the present study that back-calculated $\%CD4^+$ T-cells obtained from the existing double tube FACSCount has strong correlation against the DP FACSCalibur. The linear regression plot of back-calculated $\%CD4^+$ T-cell showed a very

good correlation indicating that the existing double tube FACSCount for obtaining back-calculated %$CD4^+$ T-cells is highly comparably with the %$CD4^+$ T-cells obtained directly from DP FACSCalibur. When the mean of the two systems was plotted against the mean difference between the two systems, the Bland-Altman bias plot, showed only a bias of +1.6% with 102% percent similarity indicating that both systems show good agreement.

Regarding comparison of back-calculated %$CD4^+$ T-cell with %CD4+ T-cell obtained directly from DP FACSCalibur, only one report could be found (Teav *et al.,* 2004). These authors had shown a good agreement (r = 0.961) between the back-calculated %$CD4^+$ T-cell, obtained from an alternative DP flow cytometry (Cyflow) and FACSCount system. Compared to the report by Teav (-0.68% with 95% CI between -1.14% and -0.216%, the present study has higher bias (+1.6%) with 95% CI between -2.3% to -4.2%. The difference in bias seen between Teav et al., (2004) and the present study might be that the study subjects in the present study are pediatric age group in which the $CD4^+$ T-cell range is known to be wide.

Regarding absolute $CD4^+$ T-cell correlation using the existing double tube FACSCount, the present study has shown strong correlation against the DP FACSCalibur (r = 0.954). When the mean of the two systems was plotted against the mean difference between the two systems the bias plot showed +29.7% having percent similarity more than 97% with CV less than 10% indicating that both systems show good agreement. It was further analyzed for absolute $CD4^+$ T-cell determination based on their immunological criteria. A lower correlation was observed in children whose absolute $CD4^+$ T-cell counts were below 200 (r = 0.765; bias -7.8 cells/µl) and between 200 and 350 cells/mm^3 (r =0.755, bias and 14.6 cells/µl) having percent similarity more than 95. Moreover, a CV of 16.3% in case of $CD4^+$ T-cell count <200 cells/mm^3 was observed indicating that the two systems vary more than 10%. In samples with absolute $CD4^+$ T-cell counts below 200 the allowable variation (CV) is within 15% (IQA, 2006).

In relation to correlation between absolute $CD4^+$ T-cell values obtained from double tube FACSCount and DP FACSCalibur system, there is no similar study conducted although a comparison of flow cytometry, assays using FACSCalibur in DP and SP system for absolute $CD4^+$ T-cells enumeration that found strong correlation (r = 0.965) was found (Jeganathan *et al.,* 2008).

Similarly comparison of absolute $CD4^+$ and $\%CD4^+$ T-cells using SP alternative flow cytometry (Cyflow ^{green}) and DP FACSCalibur system on 229 samples showed good correlation and agreement between the two methods (Manasa *et al.,* 2007). Also an evaluation of absolute $CD4^+$ T-cell values from SP alternative flow cytometry (Cyflow ^{green}) and SP FACSCount and FACSCan (TruCOUNT) showed good method agreement (Pattanapanyasat *et al.,* 2005). Although the studies showed good correlation, an alternative flow cytometry which was not the gold standard flow cytometry was used in the study. Pattanapanyasat et al (2008) performed an evaluation study that involved comparison of absolute $CD4^+$ T-cell count and $\%CD4^+$ T-cell values obtained from the double tube FACSCount and DP FACSCan system and showed a higher correlation (r = 0.978) of absolute $CD4^+$ T-cell values (Pattanapanyasat *et al.,* 2008).

For a precise and accurate absolute $CD4^+$ T-cell count dispensing of reagents and blood samples in all pipetting steps is one of the criteria of being precise and accurate in SP approaches. In contrast the DP approaches require additional value from hematology analyzer to generate absolute $CD4^+$ T-cell values, which is believed to introduce some error in the determination is a less precise approach (Deems *et al.,* 2004).

FACACalibur system which is the gold standard has an expensive initial cost than FACSCount (single platform) system. For a sustainable pediatric ART monitoring, FACSCount system is the most commonly used platform (van de Perre *et al.,* 1998). It has two replaceable software's that are the double tube and the new single tube FACSCount

which are very simple-to-use systems that require a lesser time and training than FACSCalibur.

To expand HIV monitoring facilities and to increase access to %$CD4^+$ T-cell determination for pediatric patients BD has recently developed a new single tube FACSCount. It is shown in the present study that absolute CD4+ T-cell count and %$CD4^+$ T-cells obtained from the new single tube SP FACSCount system and the SP FACSCalibur have strong correlation (0.976 and 0.983) with an overall bias +21% and +1.7%, indicating that the two systems are in good agreement. A similarly strong correlation was observed in adult population too (r = 0.970 and r = 0.960) with bias -10 % and -1.2%.

The new single tube FACSCount system produced good precision (CV is 2.0). A very strong correlation was observed between %$CD4^+$ T-cell obtained from the new single tube FACSCount with FACSCalibur which indicates that both systems showed more than 96% similarity. This finding agrees with the only report by Pattanapanyasat and colleagues (2008) in Thailand revealing an excellent correlation of absolute $CD4^+$ T-cell (r = 0.986) and %$CD4^+$ T-cell count (r = 0.977), respectively. According to the Pattanapanyasat and colleagues the mean difference of %$CD4^+$ T-cells obtained from the new BD single tube FACScount and FACSCalibur/FACScan system is -0.36% with 95% CI between -2.5% to +1.8%. In the present study the mean difference is 1.7% with 95% CI between -5.2% to +1.5% having percent similarity 96.5%. Comparing to what Pattanapanyasat and colleagues reported there is no significant difference since the upper limit is within the confidence interval reported by them. But the small difference observed might be due to the preanalytical step and technical differences in CD4 enumeration methodologies, in addition the two study groups are found in different geographical location which might affect the level of $CD4^+$ T-cells (Kassu *et al.*, 2001), otherwise both studies have showed similar results.

The newly produced single tube FACSCount system, which gives a simultaneous absolute $CD4^+$ and %$CD4^+$ T-cell count, is a very good system. On top of its best facility the existing

FACSCount system requires 60 min for incubation and 2-3 minutes to give result and can run 50 samples per day. But the new BD FACScount system requires shorter incubation (30-40 second) period and less-than-a-minute to draw the results and it is capable of running 100 samples per day. Due to the burden of high number of HIV infected children in resource poor settings, the facilities that give $CD4^+$ T-cell enumeration are under high pressure. To minimize this pressure, facilities must think of using time and effort saving system so as to hasten the operation.

The new single tube FACSCount system contains a combination of a nuclear DNA fluorescent dye and three fluorescent conjugated monoclonal antibodies that identify CD4 positive cells and CD4 negative cells. Monoclonal antibody to CD14 recognizes a human monocyte/macrophage antigen expressed on the majority of the peripheral blood monocytes, whereas monoclonal antibody to CD15 recognizes a human myelomonocytic antigen expressed on more than 95% of mature peripheral eosinophils and neutrophils and at low density on circulating monocytes. T-lymphocytes are identified by their DNA fluorescence and size while strictly excluding the non-lymphocytes from their CD14/CD15 expression; consequently $\%CD4^+$ T-cell was counted automatically. The absolute number of $CD4^+$ T-lymphocyte was calculated by multiplying the ratio of fluorescent $CD4^+$ T-lymphocyte events by the known number of reference microbeads in tube. Therefore due to this reason the new single tube FACSCount is accurate for absolute and $\%CD4^+$ T-cell enumeration.

The new single tube FACSCount system is an improvement especially for pediatric ART monitoring. It is known that FACSCalibur is the preferred flow cytometer for pediatric immunological monitoring but it has an expensive initial and maintenance cost. Since utilization of FACSCalibur system in resource limited settings (like Ethiopia) is limited due to the presence of very limited number of facilities and the need to have trained human resource, the ART services use the FACSCount system to enumerate $CD4^+$ T-cells for initiation and monitoring of ART. Consequently a cheaper and accurate $CD4^+$ T-cell determination technology is needed to alleviate initiation and monitoring of ART. Absolute

$CD4^+$ T-cell count is not as reliable in pediatrics ART management as $\%CD4^+$ T-cell value is and according to this study the back-calculated $\%CD4^+$ T-cells value usage can be used as a back-up for the new single tube $\%CD4^+$ T-cell determination.

In the present study it was not possible to test and correlate a single blood sample for absolute $CD4^+$ and $\%CD4^+$ T-cells by using all four methodologies (the double tube, single tube FACSCount, FACSCalibur TriTEST and FACSCalibur TruCOUNT systems).

6 Conclusions

1 The back-calculated %$CD4^+$ T-cell correlated well with the %$CD4^+$ T-cells from FACSCalibur TriTEST^{TM}, therefore back-calculation can be used as pediatric monitoring tool in areas where the FACSCalibur system could not be established. It should be included in the ART Guidelines for Pediatrics so that it will be used for national program. Besides it is useful to keep the back-calculation technique as a backup for %$CD4^+$ T-cell determination.

2 The new single tube FACSCount system for determination of %$CD4^+$ T-cell is well correlated with the FACSCalibur TruCOUNT ^{TM} system. This study has proved the successful use of the new single tube FACSCount system and believes that the access to initiate and monitor ART for children will improve dramatically. Therefore, the new single tube FACSCount system can be used for pediatric ART monitoring in the country but until the new system totally replace the existing FACSCount, back-calculation can be used.

7 Recommendation

This study would like to recommend that it is useful to see the correlation and agreement of absolute $CD4^+$ and %$CD4^+$ T-cells in all the systems (double tube FACSCount system, single tube FACSCount system, FACSCalibur Tritest and FACSCalibur TruCOUNT tubes system) using a pediatric population.

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9 Annexes

Annex 1. WHO Criteria for Starting ART in Children and Infants (FMOH, 2007)

Clinical Stage	Immunologic marker	Recommendation
Stage IV*	CD4	Treat all
	No CD4	Treat all
Stage III*	CD4	Treat all In children >12 months with TB** OHL, LIP, thrombocytopenia, CD4 guided.
	No CD4	Treat all
Stage II	CD4	Treat if CD4 is: <25% or <500 cells/mm^3 for infant <11 months <20% or <750 cells/mm^3 for child 12 -35 months <15% or <350 cells/mm^3 for child 36 -59 months <15% or <200 cells/mm^3 for child >5 years
	No CD4	Treat if TLC is: <4000 cells/mm^3 for infant <11 months <3000 cells/mm^3 for child 12-35 months <2500 cells/mm^3 for child 36-59 months <2000 cells/mm^3 for child 5-8 years
Stage I	CD4	Treat if CD4 is: <25% or <1500 cells/mm^3 for infant <11 months <20% or <750 cells/mm^3 for child 12-35 months <15% or <350 cells/mm^3 for child 36-59 months <5% or <200 cells/mm^3 for child >5 years
	No CD4	Do not treat
Presumptive severe HIV disease		Treat all irrespective of CD4 value

*Stabilize any OI before initiating ART.
**Pulmonary TB: Evaluate possibility to defer initiation of ART depending on assessment of clinical status and CD4, and clinical and immunological response to TB therapy.

Annex 2. (a)- ARV drugs available nationally (FMOH, 2008) and **(b)-** ARV drugs available for infants as of 2006 internationally (Orne-Gliemann *et al*., 2006)

a

Child 1st line regimen	
1-3 years	d4T+3TC+NVP or AZT+3TC+NVP
≥3 year s	d4T+3Tcplus NVP or EFV# or AZT+3TC plus NVP or EFV#
# EFV should be avoided in post puberty girls who are either in the 1st trimester or pregnancy or are sexually active and should not using adequate contraceptive	
Infants up to 12 months with no history of PMTCT or NNRTI exposure*	d4T+3TC+NVP* or AZT+3TC+NVP*
Infants up to 12 months with no history of PMTCT or NNRTI exposure*	D4T+3TC+Kaletra** or AZT+3TC+Kaletra**
* For HIV infected infants with a history of exposure to single dose neverapin or non-nucleoside reverse transcriptase inhibitor containing maternal antiretroviral therapy or preventive antiretroviral regimen, a protease inhibitor- based triple antiretroviral therapy regimen should be started ** Where protease inhibitor are not available, or feasible, neverapin-based therapy should be used	
Child 2nd line regimen	
	ddl+ABC+ LPV/r* ddl+ABC+NFV
* NFV can be substituted for LPV/r if no cold chain is available	

b

NRTIs (Nucleoside Reverse transcriptase Inhibitors)

- Combivir (ZDV/3TC)
- Emtricitabine (FTC)
- **Lamivudine (3TC)**
- **Zidovudine (ZDV/AZT)**
- Zalcitabine (ddC)
- Trizivir (ABC/ZDV/3TC)
- **Didanosine (ddI)**
- **Tenofovir (TDF)**
- **Stavudine (d4T)**
- **Abacavir (ABC**)

NNRTIs (Non-nucleoside Reverse transcriptase Inhibitors)

- **Nevirapine (NVP)**
- **Efavirenz (EFV)**

Protease Inhibitors

- Amprenavir
- Indinavir
- Saquinavir

Lopinavir/Ritonanvir (LPV/r)

- Fosamprenavir
- Atazanavir

Nelfinavir (NFV)

Annex 3. WHO clinical staging of HIV/AIDS for infants and children with confirmed HIV infection (WHO, 2007)

Clinical stage	Clinical event	Clinical diagnosis	Definitive diagnosis
1	Asymptomatic	No HIV-related symptom, no clinical sign on examination	Not applicable
	Persistent Generalized Lymphdenopathy	Persistent enlarged lymph nodes >1cm	Clinical diagnosis
2	Unexplained persistent hepatosplenomegaly	Enlarged liver and spleen without any cause	Clinical diagnosis
	Papular pruritic eruptions	Popular pruritic vesicular lesions	Clinical diagnosis
	Extensive wart virus infection	Characteristic warty skin lesions; small fleshy grainy bumps, often rough, flat on sole of feet (plantar warts);facial, more than 5% of body area or disfiguring.	Clinical diagnosis
	Extensive molluscum contaginosum	Characteristic skin lesions: small flesh-coloured, pearly or pink, dome-shaped or umbilicated growths, may be inflamed or red; facial, more than 5%of body area or disfiguring. Giant molluscum may indicate advanced immunodeficiency.	Clinical diagnosis
	Fungal nail infections	Fungal paronychia (painful, red and swollen nail bed)or onycholysis (painless separation of the nail from the nail bed).Proximal white subungual onchomycosis is uncommon without immunodeficiency	Clinical diagnosis
	Recurrent oral ulcerations	Aphthous ulceration, typically with a halo of inflammation and yellow-grey pseudomembrane.	Clinical diagnosis
	Lineal gingival erythema (LGE)	Erythematous band that follows the contour of the free gingival line; may be associated with spontaneous bleeding.	Clinical diagnosis
	Angular cheilitis	Splits or cracks on lips at the angle of the mouth with depigmentation, usually responding to antifungal treatment but may recur	Clinical diagnosis
	Unexplained persistence of parotid enlargement	Asymptomatic bilateral swelling that may Spontaneously resolve and recur in absence of other known cause; usually painless.	Clinical diagnosis

	Herpes zoster	Painful rash with fluid-filled blisters, dermatomal distribution, may be haemorrhagic on erythematous background, and may become large and confluent. Does not cross the midline.	Clinical diagnosis
	Recurrent or chronic upper respiratory tract infection (otitis media, otorrhoea, sinusitis, tonsillitis	Current event with at least one episode in past six months. Symptom complex: fever with unilateral face pain and nasal discharge (sinusitis)or painful swollen eardrum (otitis media), sore throat with productive cough (bronchitis), sore throat (pharyngitis) and barking croup-like cough (LTB),persistent or recurrent ear discharge.	Clinical diagnosis
3	Unexplained moderate malnutrition not adequately responding to standard therapy	Weight loss: low weight-for-age, up to -2 standard deviations (SDs), not explained by poor or inadequate feeding and/or other infections, and not adequately responding to standard management.	Documented loss of body weight of-2SDs, failure to gain weight on standard management and no other cause identified during
	Unexplained persistent diarrhea (14 days or more)	Unexplained persistent (14 days or more) Diarrhoea (loose or watery stool, three or more times daily) not responding to standard treatment.	Stools observed and documented as unformed. Culture and microscopy reveal no pathogens.
	Unexplained persistent fever (above 37.5oC, intermittent or constant, for longer than one month)	Reports of fever or night sweats for longer than one month, either intermittent or constant, with reported lack of response to antibiotics or antimalarials. No other obvious foci of disease reported or found on examination. Malaria must be excluded in malarious areas.	Documented fever of >37.5 oC with negative blood culture, negative malaria slide and normal or unchanged CXR, and no other obvious foci of disease.
	Oral candidiasis (after first 6 weeks of life)	Persistent or recurring creamy white soft small plaques which can be scraped off (pseudomembranous),or red patches on tongue, palate or lining of mouth, usually painful or tender (erythematousform).	Microscopy or culture

	Oral hairy leukoplakia	Fine small linear patches on lateral borders of tongue, generally bilaterally, which do not scrape off.	Clinical diagnosis
	Acute necrotizing ulcerative gingivitis/ periodontitis	Severe pain, ulcerated gingival papillae, loosening of teeth, spontaneous bleeding, bad odour, and rapid loss of bone and/or soft tissue.	Clinical diagnosis
	Lymph node Tuberculosis	Non acute, painless "cold "enlargement of lymph nodes, usually matted, localized in one region. May have draining sinuses. Response to standard anti-TB treatment in one month.	History or fine needle aspirate for Zeihl Nielson stain. Culture
	Pulmonary Tuberculosis	Nonspecific symptoms, e.g. chronic cough, fever, night sweats, anorexia and weight loss. In older children, productive cough and haemoptysis as well.	Isolation of *M. Tuberculosis* on sputum culture,+/- Abnormal reaction
	Severe recurrent bacterial pneumonia	Cough with fast breathing, chest in drawing, nasal flaring, wheezing, and grunting. Crackles or consolidation on auscultation. Responds to course of antibiotics. Current episode plus one or more in previous six months.	Isolation of bacteria from appropriate clinical specimens (induced sputum, BAL, lung aspirate).
	Symptomatic lymphoid interstitial pneumonities	No presumptive clinical diagnosis	bilateral reticulonodular interstitial pulmonary infiltrates present for more than two months with no response to antibiotic treatment and no other pathogen found. Oxygen saturation persistently <90%. May present with cor pulmonale and may have increased exercise-induced fatigue. Characteristic histology.
	Chronic HIV-associated lung disease	History of cough productive of copious amounts of purulent sputum (bronchiectasis only), with or without clubbing, halitosis, and crepitations and/or wheezes on auscultation.	may show honeycomb appearance (small cysts) and/or persistent areas of opacification and/or widespread lung destruction, with fibrosis and loss of volume.
	Unexplained anaemia (<8g/dl), and or neutropenia (<0.5X10^9/L) and or Chronic thrombocytopenia (<50X 10^9/L)	No presumptive clinical diagnosis	Laboratory testing, not explained by other non-HIV conditions, or not responding to standard therapy with haematinics, antimalarials or anthelminthics as outlined in IMCI.

4	Unexplained severe wasting or severe malnutrition not adequately responding to standard therapy	Persistent weight loss not explained by poor or inadequate feeding or other infections and not adequately responding in two weeks to standard therapy. Characterized by: visible severe wasting of muscles, with or without oedema of both feet, and/or weight-for-heightof-3SDs, as defined by WHO IMCI guidelines.	Documented weight loss of >-3 SD +/-oedema.
	Pneumocystis pneumonia	Dry cough, progressive difficulty in breathing, cyanosis, tachypnoea and fever; chest in drawing or strid or. (Severe or very severe pneumonia) Usually of rapid onset especially in infants under 6 months of age. Response to high-dose co-trimoxazole +/-prednisolone.	typical bilateral perihilar diffuse infiltrates; microscopy of induced sputum
	Recurrent severe bacterial infections (e.g. empyema, pyomyositis, bone or joint infection, meningitis, but excluding pneumonia)	Fever accompanied by specific symptoms Or signs that localize infection. Responds to antibiotics. Current episode plus one or more in previous six months.	Culture of appropriate clinical specimen.
	Chronic herpes simplex infection ; (orolabial or cutaneous of more than one month's duration or visceral at any site)	Severe and progressive painful orolabial, genital, or anorectal lesions caused by herpes simplex virus infection present for more than one month.	Culture and/or histology.
	Extrapulmonary Tuberculosis	Systemic illness usually with prolonged fever, night sweats, and weight loss. Clinical features of organs involved, e.g. sterile pyuria, pericarditis, ascites, pleural effusion, meningitis, arthritis, orchitis.	Positive microscopy showing acid fast bacilli or culture of *M. tuberculosis* from blood or other relevant specimen except sputum or Biopsy and histology.
	Kaposi's sarcoma	Typical appearance in skin or oropharynx Of persistent, initially flat, patches with a pink or blood-bruise colour, skin lesions that usually develop into nodules.	Macroscopic appearance or by histology.

	Oesophageal candidiasis (or Candida of trachea, bronchi or lungs)	Chest pain and dysphagia (difficulty in swallowing), odynophagia (pain on swallowing food and fluids) or retrosternal pain worse on swallowing (food and fluids) responds to specific treatment. In young children, suspect particularly if oral Candida observed and food refusal occurs and/or difficulties/crying when feeding.	Macroscopic appearance at endoscopy, microscopy of specimen from tissue or macroscopic appearance at bronchoscopy or histology.
	Central nervous system toxoplasmosis (after the neonatal period)	Fever, headache, focal neurological signs, convulsions. Usually responds within 10 days to specific therapy.	Positive serum toxoplasma antibody AND of available single/multiple intracranial mass lesions on neuroimaging
	HIV encephalopathy	At least one of the following, progressing over at least two months in the absence of another illness: – failure to attain, or loss of, developmental milestones, loss of intellectual ability; or – progressive impaired brain growth demonstrated by stagnation of head circumference; or – acquired symmetric motor deficit accompanied by two or more of the following: paresis, pathological re .exes, ataxia, gait disturbances.	Neuro imaging demonstrating atrophy and basal ganglia calcification, exclusion of other causes.
	Cytomegalovirus (CMV) infection; retinitis or CMV infection affecting another organ, with onset at age over 1month	Retinitis only: may be diagnosed by experienced clinicians: typical eye lesions on fundoscopic examination; discrete patches of retinal whitening with distinct borders, spreading centrifugally, often following blood vessels, associated with retinal vasculitis, haemorrhage and necrosis.	Histology or Cytomegalovirus demonstrated in CSF by culture or DNA-PCR.
	Extrapulmonary cryptococcosis including meningitis	Meningitis: usually subacute, fever with increasing severe headache, meningism, confusion, behavioural changes that responds to cryptococcal therapy.	Isolation of Cryptococcus neoformans from extrapulmonary site or positive cryptococcal antigen test (CRAG) on CSF or blood.

	Disseminated mycosis (extrapulmonary histoplasmosis, coccidiomycosis)	No presumptive clinical diagnosis. Histology: usually granuloma formation.	Isolation: antigen detection from affected tissue; culture or microscopy from clinical specimen or blood culture.
	Chronic cryptosporidiosis (with diarrhea)	No presumptive clinical diagnosis.	Cysts identified on modified ZN stain.
	Chronic isospoiasis	No presumptive clinical diagnosis.	Identification of isospora.
	Disseminated non-tuberculous mycobacteria infection	No presumptive clinical diagnosis.	Nonspecific clinical symptoms including progressive weight loss, fever, anaemia, night sweats, fatigue or diarrhoea; plus culture of atypical mycobacteria species from stool, blood, body fluid or other body tissue, excluding lung.
	Cerebral or B cell non-Hodgkin lymphoma	No presumptive clinical diagnosis.	CNS imaging: at least one lesion with mass effect; histology of relevant specimen.
	Progressive multifocal leukoencephalopathy (PML)	No presumptive clinical diagnosis.	Progressive neurological disorder together with white matter lesions on neuroimaging or positive polyomavirus JC (JCV) PCR on CSF.
	Symptomatic HIV-associated cardiomyopathy	No presumptive clinical diagnosis.	Cardiomegaly and evidence of poor left ventricular function confirmed by chocardiography
	Symptomatic HIV-associated nephropathy	No presumptive clinical diagnosis	Renal biospy

i unexplained refers to where the condition is not explained by other causes.

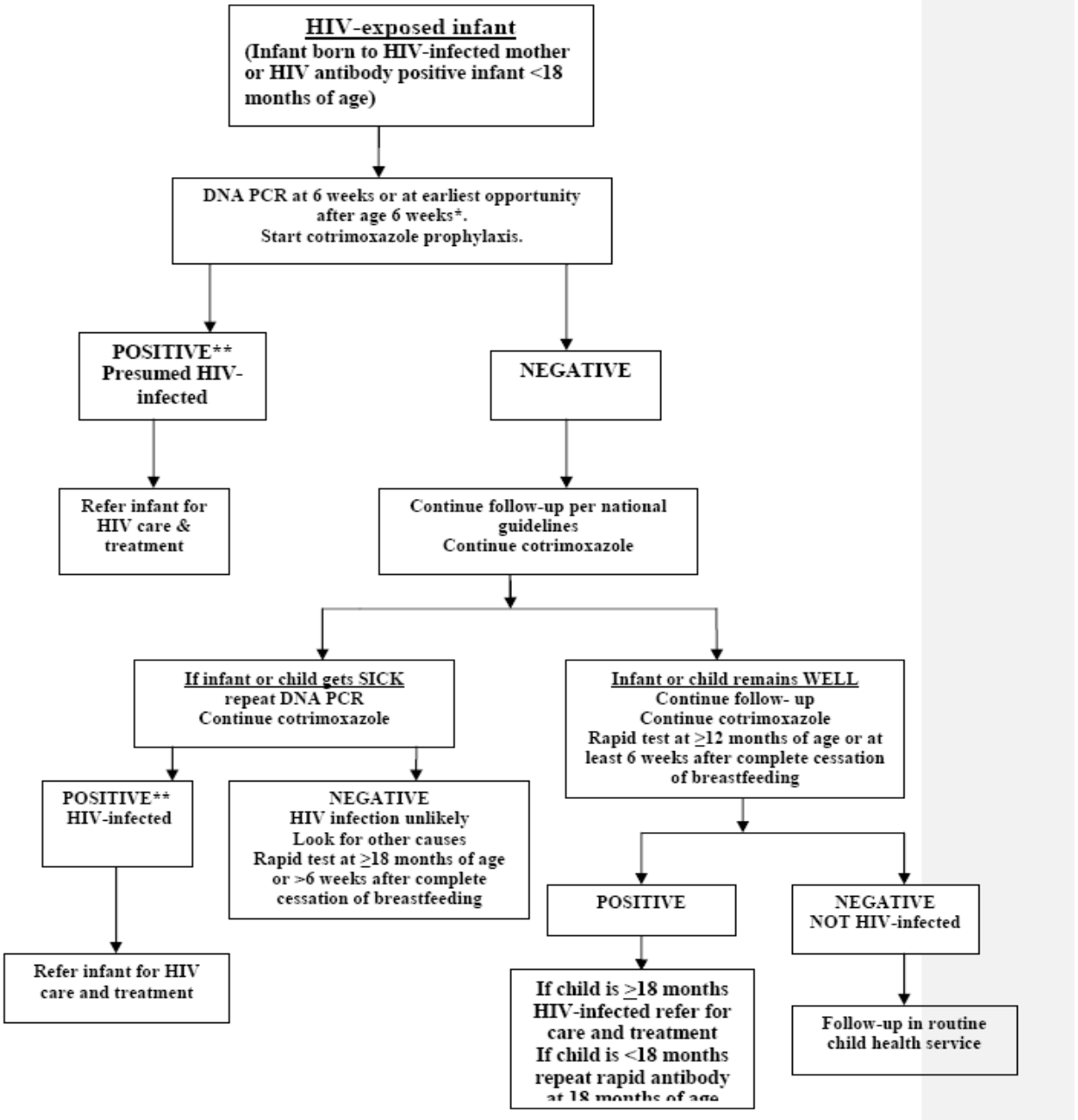
ii some additional specific conditioned can be included in regional classifications (e.g. Penicilliosis in Asia, HIV associated rectovaginal fistula in Africa)

Annex 4. Age-specific immunologic category of absolute $CD4^+$ and % $CD4^+$ T cell (CDC, 1994; WHO, 2006)

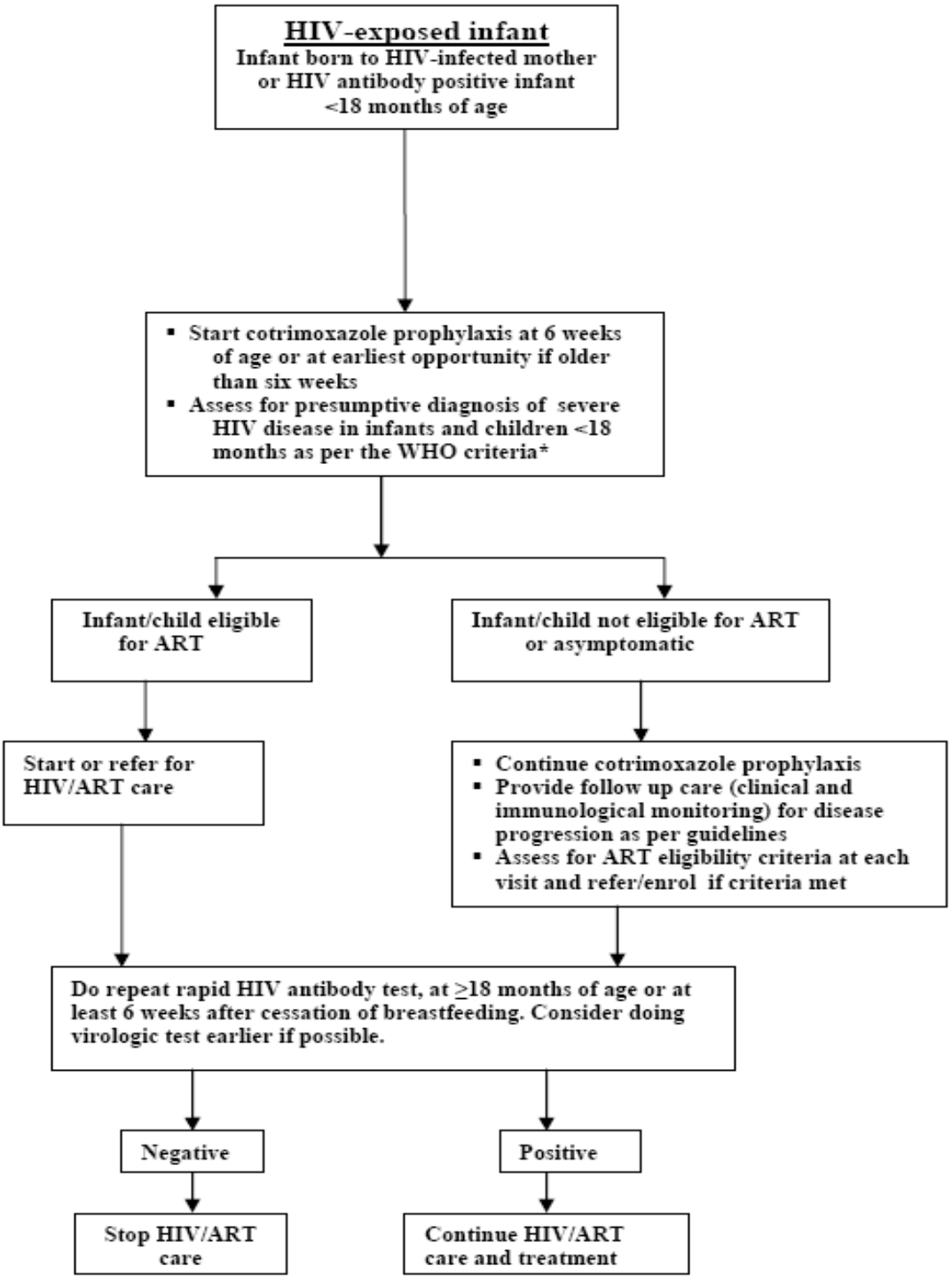
A. Immunological classification for children <13 years of age							
Immunological category		**<12 months**		**1-5 years**		**6-12 years**	
		abs.	**%**	**abs.**	**%**	**abs.**	**%**
1	No suppression	≥1,500	≥25	≥1,000	≥25	≥500	≥25
2	Moderate suppression	750-1,499	15-24	500-999	15-24	200-499	15-24
3	Severe suppression	<750	<15	<500	<15	<200	<15

B. Immunological classification for adolescent >13 years of age			
1	No suppression	≥500	22.5-35
2	Moderate suppression	200-499	14-22
3	Severe suppression	<200	<14

Annex 5a. National Algorithm. HIV testing in HIV-exposed infants <18 months where virological testing is available (Ethiopia Federal HIV/AIDS Prevention and Control Office Federal Ministry of Health, July 2008)



Annex 5b. National Algorithm. Diagnostic algorithm for infants <18 months of age where virological tests are unavailable (Ethiopia Federal HIV/AIDS Prevention and Control Office Federal Ministry of Health, 2007)



Annex 6. Clinical criteria for presumptive diagnosis of severe HIV disease among infants and children aged <18 months in situations where virologic testing is not available (WHO, 2006)

A presumptive diagnosis of severe HIV disease should be made if:

- The infant is confirmed as HIV-antibody positive AND
- Diagnosis of any AIDS-indicator conditions [a] can be made OR
- The infant is symptomatic with two or more of the following;
  - Oral thrush
  - Sever pneumonia
  - Severe sepsis

Other factors that support the diagnosis of severe HIV disease in an HIV-seropositive infant include:

- Recent HIV-related maternal death or advanced HIV disease in the mother
- CD4 <20%

Confirmation of the diagnosis of HIV infection should be sought as soon as possible

a. conditions such as clinical stage 4 diseases

Annex 7. Follow up schedule for HIV-infected children (Ethiopia Federal HIV/AIDS Prevention and Control Office Federal Ministry of Health, July 2008)

Follow-up schedule for HIV-infected children	
Age	Visit interval*
0-12 months	Monthly
12-24 months on ART	Monthly
>24 months pre-ART	Every 2-3 months if Asymptomatic, monthly if sympthomatic
>24 months on ART	Every 2-3 months
*This is the minimum; children should be seen more frequently if clinically indicated **Children >24 months should be seen monthly for the first 6 months after initiation of ART then every 2-3 months if stable	