EVALUATION OF POINT-OF-CARE TEST FOR EARLY INFANT DIAGNOSIS AND VIRAL LOAD MONITORING OF HIV-1 INFECTION IN WESTERN KENYA

BY

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DECLARATION

Declaration by the student

I declare that this thesis has not been previously presented for a degree in Maseno University or any other University. I have carried out the work reported herein and all sources of information have been acknowledged by means of references.

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DEDICATION

This work is dedicated to my parents Cerina Kita and the late Stanley Ouma,my lovely wife LilianAdhiambo, Son Stanley Ouma and daughter Saskia Kita for the support they provided during the study period.

ABSTRACT

In developing countries with disproportionately high burden of HIV-1 (HIV) infections, access to early infant diagnosis (EID) and viral load (VL) HIV nucleic acid testing is limited by the prohibitive infrastructural costs of centralized testing laboratories. To increase access to HIVEID and VL monitoring, development of robust and low cost point of care (POC) technologies is a priority of current HIV diagnostic research. In Kenya, a number of POC technologies are being evaluated for EID and VL. One such POC technology is the simple amplification based assay (SAMBA) which is most advanced in its validation for use in a number of countries. However, in Western Kenya, with highest HIV prevalence, and possibly a wide range of HIV subtypes and recombinant forms, the performance of SAMBA has not been determined. The objective of the study was to evaluate the laboratory performance indices of SAMBA on HIV EID and VL testing. The study was cross sectional conducted in patient support facilities in 6 counties within Western Kenya region (namely Siaya, Nyamira, Kisii, Kisumu, Migori and Homabay counties). Male and female participants were recruited into the study and were distributed as follows: Infants aged <18 months (n=335) and adults aged >18 years (n=200). Patients were randomly recruited from the six counties. Whole blood from infants and plasma (separated at the facilities) from adults were collected from the facilities and shipped to the KEMRI/CDC Kisian testing laboratory. Whole blood were tested on SAMBA-qualitative (SAMBA-Q) assay for EID and plasma on SAMBA-semi-quantitative assay (SAMBA-SQ) for VL. All specimens were tested in parallel on the reference standard of care CobasAmpliprep/Taqman (CAP/CTM) assay. Specimens (n=11) with discordant results were further analyzed on Abbott m2000 as a tie breaker. In addition, total RNA was extracted from the discordant specimens, HIV *pol* gene amplified by RT-PCR and Nested PCR and genotyped by an in-house assay to determine the HIV subtypes. SAMBA-Q showed sensitivity, specificity and concordance of 100% (95% CI: 98.2-100), 99.3% (95% CI: 95.9-99.9) and Cohen Kappa of 0.99 (95% CI: 0.98-1.00) respectively. Similarly, SAMBA-SQ showed sensitivity, specificity and concordance of 92.0% (95% CI: 84.8, 96.5), 98.0% (95% CI: 93.0, 99.8) and Cohen Kappa of 0.90 (95% CI: 0.84-0.96) respectively. The frequency of discrepancies between SAMBA and CAP/CTM was 2% (11/535).Among the 10successfully genotyped specimens 60% (n=6)were circulating recombinant forms (CRFs) AD(n=5)and AC(n=1) while 40% were pure subtype A1 (n=3)and A(n=1).SAMBA-Q and SAMBA-SQ had comparable accuracy and reliability to CAP/CTM in detecting and quantifying HIV hence should be considered for adoption as a POC to increase access to EID and VL testing in Western Kenya.

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LIST OF ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ART	Anti-Retroviral Therapy
AVL	Viral Lysis Buffer
AW1	Wash Buffer 1
AW2	Wash Buffer 2
AVE	Elution Buffer
SAMBA	Simple Amplification Based Assay
CAP	Cobas Ampliprep
CCR5	C-C Chemokine Receptor type 5
cDNA	Complimentary Deoxyribonucleic Acid
CDC	Centers for Disease Control and Prevention
CD4+T	Cluster of Differentiation 4
CRFs	Circulating Recombinant Forms
CTM	Cobas Taqman
CXCR4	C-X-C Chemokine Receptor type 4
DBS	Dried Blood Spot
DNA	Deoxyribonucleic Acid
EID	Early Infant Diagnosis
FDA	Food and Drug Administration
HIV	Human Immunodeficiency Virus
KAIS	Kenya AIDS Indicator Survey
KEMRI	Kenya Medical Research Institute
LTFU	Loss To Follow Up
LTR	Long Terminal Repeats
MEGA	Molecular Evolutionary Genetic Analysis
NASBA	Nucleic Acid Sequence Based Amplification
NAT	Nucleic Acid Testing
POC	Point of Care
PMTCT	Prevention-of Mother To Child Transmission
Q	Qualitative

RLS	Resource-limited Settings
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SQ	Semi-Quantitative
SQUAT	Sequence Quality Assessment Tool
UNAIDS	United Nations Programme on HIV/AIDS
SQ	Semi-Quantitative Forms
VL	Viral Load
WHO	World Health Organization
µL	Microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Acquired Immunodeficiency Syndrome (AIDS) due to HIV infection is the leading cause of death in sub-Saharan Africa and the fourth major cause of mortality worldwide (UNAIDS, 2014b; UNAIDS, 2014c).Globally, an estimated 35.3 (32.2–38.8) million people were living with HIV in 2013 with 2.3 (1.9–2.7) million new infections reported (UNAIDS, 2014b). According to United Nations Programme on HIV/AIDS (UNAIDS, 2013b), over 60% of the world's HIV-infected people live in Africa; with over half of infected adults being women of child bearing age (UNAIDS, 2014b). With theadvent and scale up of prevention of mother to child transmission (PMTCT) program,increased access by HIVinfected pregnant women hasyielded encouraging results with over 60% of pregnant women in sub-Saharan Africa accessing some level of PMCT before delivery. This has led to a reduction in the number of new infections. However, efforts to identify HIV-infected infants have been less successful due to limited access to testing facilities having nucleic acid testing (PCR)capacity (UNAIDS, 2012). Thereare a number of tests available for the diagnosis of HIV infection. These include HIV antibody tests, p24 antigen tests and PCR-based tests(UNAIDS, 2012). Of these, HIV antibody tests are most commonly used for routine diagnosis of patients older than 18 months of age because they are inexpensive and accurate when performed correctly(UNAIDS, 2012). Despite the availability of rapid the tests, for HIV exposed infants,passively acquired antibodies to HIV from mothers are persistent in infants until 18 months of age (WHO, 2013). In addition, maternal and infant self-generated antibodies (IgGs) cannot be distinguished from each other (WHO, 2013). Therefore, in HIV exposed infants below age 18 months, HIV infection cannot be accurately diagnosed with conventional antibody-based detection assaysdue to possible high false positive diagnosis (WHO, 2013). Instead nucleic acid testing (DNA PCR or RNA PCR) is recommended to be used to diagnose infants in this age group(WHO, 2013)Through the existing centralized EID testing system, only one-third of HIV exposed infantsglobally who are below 18 months receive an HIV diagnostic test, with some countries reporting HIV exposed infant testing rates below 10% of eligible infant (UNAIDS, 2013b).This leaves millions of HIVexposed

infants without an initial or definitive diagnosis (UNAIDS, 2013b). Importantly, an estimated 2.5 million HIV-infected children currently in need of ART remain at risk of severe illness and death and nearly 75% of them are not currently enrolled into HIV care and treatment(WHO, 2013).Without intervention or treatment, HIV-infection in children rapidly progress to AIDS with about 35-40% and 50-60% dying before their first and second birthday respectively (Newell *et al*., 2004; WHO, 2011).Survival of these infected children depends on timely early diagnosis by nucleic acid testing (NAT) whoseaccess can be improved by adopting point of care (POC) testing and initiation of antiretroviral therapy (ART).

EffectiveHIV treatment depends on accurate immunological and virological monitoring of HIV patients (Barth *et al*., 2010). As a biomarker, CD4+T lymphocyte counts have been used guide programmatic decisions around eligibility for ART, immunological and clinical monitoring (WHO, 2013). While low CD4+Tlymphocyte counts suggest immune suppression, the relationship between CD4+ T lymphocyte counts is considerably less clear. Studies have suggested CD4+T lymphocyte to be less accurate compared to VL in HIV treatment monitoring (Rawizza *et al*., 2011; van Oosterhout *et al*., 2009). Use of CD4+ T lymphocyte may result in substantial misclassification of treatment failure potentially leading to both accumulation of drug-resistance mutations and inappropriate therapy switches to the more costly second line ART regimens (Rawizza *et al*., 2011; van Oosterhout *et al*., 2009; WHO, 2013). According to WHO 2013 guidelines and other studies, virological monitoring using viral load (VL) testing is the most reliable diagnosis of treatment failure preventing unwarranted treatment regimen switch and accumulation of drug resistant strains that may limit future treatment options (Barth *et al*., 2010; Yang *et al*., 2004; WHO, 2013).In sub-Saharan countriestargeted VL testing is through centralizedlaboratories. Therefore access to timely VL monitoring for patients on antiretroviral therapy is limited by logistical challenges includingpoor laboratory infrastructure, prohibitive costs of equipment, shipping and cold chain requirements for transportation and storage(Blyth *et al*., 2011; Fiscus*et al*., 1998; Mabey*et al*., 2004). In addition results frequently take long to get back to clinicians resulting indelayed proper management of patients experiencingvirological treatment failure. Consequently, scale up of access to ART is compromised due to increase in overall costs of care and limited treatment options (Yang *et al*., 2004).To increase access to

VLtesting, the use of dried blood spots(DBS) as an alternative to plasma has been explored. The use of DBS has several advantages including specimen collection via finger or heel stick, low biohazard risk, and ease of transport bystandard courier services under ambient conditions(Yang *et al*., 2010). Data on DBS for VLhas shown an inherentchallengewithregard to low specificity (Brambilla*et al*., 2003; Ouma *etal*., 2013).

While the new WHO guideline advocates for universal access of early infant diagnosis (EID) and use of routine VL for treatment monitoring, this is impractical in resource poor settings (WHO, 2013).Currently in KenyaEID and VL testing areperformed in only seven centralized laboratories which are sparsely distributed.These laboratories include three Kenya Medical Research Institute (KEMRI) laboratories (Kisumu, Nairobi and Busia), National Public Health Reference laboratory in Nairobi, Coast General Hospital laboratory and AMPATH care laboratory in Eldoret (KAIS, 2014). These centralized laboratories mostly use nucleic acid testing technologies which detect HIV-1 pro-viral DNA for EID and quantify circulating HIV-1 RNA for VL.These testing technologies require sophisticated instruments, proper infrastructure and trained technicians in a controlled environment. Thenucleic acid based technologies in use include Roche COBAS® AmpliPrep/COBAS® TaqMan® System (CAP/CTM) and Abbott m2000 which areReal-time PCR technologies. The CAP/CTM assay is able to co-amplify two target regions of HIV (known as the *gag* and long terminal repeat (LTR) regions), which are not current HIV drug targets(Roche, 2008). By targeting both regions of the HIV genome simultaneously, the test increases the probability of detection of virus particles despite the high rate of HIV mutation(Roche, 2008). Abbott m2000 assay has primers targeting the conserved region of *pol-integrase* genes and amplify HIV-1 RNA using a partially double-stranded qRT-PCR method(Abbott, 2007). By targeting the conserved*pol-integrase* gene, the assay aims to minimize inefficient binding due to sequence mismatch at the probe binding site (Abbott, 2007). The two assayshave higher throughput, larger dynamic ranges andautomate all extraction steps.In addition the centralized laboratories function well above capacity leading to backlogs and long turn-around times that is required to get back results to the clinicians.Due to the long turn-around time, lost-to-follow-up (LTFU) has become one of the major problems in providing effective treatment and care to HIV-1 infected patients (Braitstein*et al*., 2011; Haleyur*et al*.,

2014; Katzenstein*et al*., 1995). Given the limitations of laboratory-based testing, feasible alternatives that can improve access and reduce the cost of EID and VL testing in resource-limited settings needs to be adopted and brought closer to the point of patient care. POCtesting provides a potential alternative to overcome some of these challenges facilitating early detection and treatment of HIV. This will subsequently enhance preventive impact of ART and help to ensure an appropriate and rapid response to emergence of drug resistance, a problem likely to grow substantially over the coming years(Laursen *et al*., 2012). In addition to the infrastructural challenges of centralized testing, the performance of HIV-1 moleculardiagnosis and monitoring tests is complicated by high HIV-1 genetic diversity in different populationsinfected with various subtypes including circulating and unique recombinant forms(Katzenstein*et al*., 1995; Korn*et al*., 2009; Luft*et al*., 2011; Swanson *et al*., 2005; Taylor *et al*., 2008). This has led to false negatives and under estimationof VL by commercial assays (Alaeus *et al.,* 1997*;* Korn *et al.,* 2009*;* Wirden *et al*., 2009).It is therefore essential to continue evaluating these assays against multiple HIVsubtypes to ensure accurate diagnosis and VLtesting in settings with high prevalence of diverse HIV-1 subtypes. There is therefore a growing needto design simple, affordable, robust and accurate point of care testing technologies. One such POC nucleic acid assay is the Simple Amplification Based Assay (SAMBA) that can perform both qualitative HIV diagnostic tests (SAMBA-Q early infant diagnosis) and semi-quantitative HIV VL tests (SAMBA-SQ) distinguishing between patients with viral copies above or below the clinical threshold of 1000 cp/ml.

Diagnostics Development Unit (DDU) of University of Cambridge developed SAMBA(Lee *et al*., 2008, Ritchie *et al*., 2014). In November 2013, through collaboration with KEMRI/CDC,SAMBAwas introduced for an in-countryevaluationto inform its adoption in Kenya. In contrast to conventional nucleic acid tests (NATs), SAMBA assay relies on Helicase-Amplification that is an isothermal amplification technology. This technology allows the SAMBA to operate at a single temperature eliminating the need for a thermocycler enabling assays to be conducted on simple and portable heating systems. Nucleic acids are extracted utilizing non-toxic aqueous-based method and amplified with primers targeting the conserved long terminal repeat regions of HIV-1 (Lee*et al*., 2008). The amplification is completed within

60 minutes. The amplified HIV-1 nucleic acid is further detected by dipstick-based detection method(Dineva*et al*., 2005).

1.2 Problem statement

To minimize challenges of centralized testing and provide efficient patient care at minimal cost, there is need for efficient, accurate and easy to performPOC tests. Such new developed POC tests like SAMBA hold promise for improved management of HIV infected patients. However like other tests, differences in population characteristics and diverse genetic variation of HIV may influence their performance. Therefore before implementation, there is need for evaluationof the tests' performance characteristics in different populations. This study intends to evaluate performance of SAMBA on HIV-1 EID and VL monitoring in Western Kenya.

1.3 Justification

Prompt knowledge of HIV status in infants and accurate monitoring of HIV clinical progression have potential health benefits leading to reduced morbidity and mortalitydue to ART. Current available assays have been mainly been validated for HIV-1 subtype B which is prevalent in developed countries. As such, these assays may perform sub-optimally when utilized in developing countries where non-B HIV-1 subtypes and recombinant forms predominate. In addition, majority of the existing HIV-1 diagnostic assays require well-developed laboratory infrastructure and properly trained technicians. To increase access to HIV diagnostic and monitoring services, development of robust and low cost technologies is a priority of current HIV research. SAMBA is among the first nucleic acid testing POC technology in Kenya that may offer feasible alternative to meet the need for an affordable and robust technology for HIV diagnosis. Currently, there is limited data on the performance of the SAMBA assay in Kenya, hence the need to evaluateits use for possible adoption by the Kenyan government. This may contribute towards decentralizationof EID and VL testing potentially improving health outcomes of HIV-1 patients.

1.4 Null Hypothesis

i. There is no difference in performancecharacteristics ofSAMBA compared toCAP/CTM in detection of HIV-1 and VLmonitoring.

ii. HIV-1 subtypes do not influence HIV-1 detection by SAMBA.

1.5 General objective

To estimate the laboratory performance indices of the SAMBA-Q and SAMBA-SQ assays compared to reference standard careCAP/CTM assay.

1.5.1 Specific objectives

i. To determine the sensitivity, specificity and concordance correlation of SAMBA-Q assay in diagnosis of HIV-1 in infants.

ii. To determine the specificity, sensitivity and concordance correlation of SAMBA-SQ assay for HIV-1 VL testing in adults.

iii. To determine the HIV-1 subtypes not detected by SAMBA.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Epidemiology of HIV

HIVcauses AIDSthat has posed world's most serious health and development challenges.HIV is a leading cause of death worldwide and the number one cause of death in Africa(Korn*et al*., 2009). The first cases were reportedon men who had sex with men as a new clinical entity in 1981 in the United States of America (CDC, 1981a; CDC, 1981b; Korn*et al*., 2009). Though initially lifestyle and behavioral factors were hypothesized to be causally related, finally in 1983 HIV was identified as the true cause of AIDS(Barre-Sinoussi *et al., 1983;* Broder and Gallo, 1984; Gallo *et al*., 1984).

By 2013, approximately 35 million people wereliving with HIV. This has increased from 29.8 million people in 2001though global prevalence rate has leveled at 0.8%.While incidences have been reported in all regions of the world, approximately 68% are in sub-Saharan Africa (UNAIDS, 2014a).The contributing factors include continuing new infections, people living longer with HIVdue to effective ART and general population growth. Approximately 1.5 million people died of AIDS in 2013representing 35% decrease since 2005(UNAIDS, 2013a; UNAIDS, 2013b; UNAIDS, 2014c).Deaths have declined partly due to the advent and scale up of antiretroviral treatment (ART).As of December 2013, only 37% (12.9 million) of people living with HIV were receiving HIV treatment, leaving more than 22 million people living with HIV without treatment. Substantial coverage gaps exist within and among regions. In Africa for example, treatment coverage in 2013 was 41% in Eastern and Southern Africa(WHO/UNAIDS/UNICEF, 2013).HIV not only affects the health of individuals, it impacts households, communities, development and economic growth of nations. Despite these challenges, in the last decade new global efforts have been implemented to address the epidemic and signs of limiting the epidemic have been recorded(WHO/UNAIDS/UNICEF, 2013). For example new HIV infections have declined globally by 38% since 2001. Although risk factors vary, most new infections are transmitted heterosexually, mother to child, men who have sex with men, injecting drug users. Although HIV testing capacity has increased over time, enabling

more people to learn their HIV status, approximately half of these people infected with HIV are still unaware they are infected(UNAIDS, 2014a).

Sub-Saharan Africa, home to only about 13% of the world's population is the hardest hit region with 71% of people living with HIV (Bureau, 2013; UNAIDS, 2013a). Most children (91%)with HIV live in this region(UNAIDS, 2014a). In Kenya, 5.6% of adults and adolescents aged 15-64 years were infected with HIV in 2013, representing an estimated 1,192,000 people. Women were more likely to be infected (6.9%) than men (4.4%). There was wide geographical variation in adult HIV prevalence ranging from 2.1% in North Eastern region to 15.1% in Nyanza region(KAIS, 2014).

2.1.1 HIV diversity and geographical distribution

HIV is a lentivirus whose isolates are classified into two major types; HIV-1 and HIV-2, the former is distributed worldwide while the later mostly common in West Africa(Robertson *et al*., 2000). Of the two types, HIV-1 is responsible for most HIV pandemic (Constantine *et al*., 2005). HIV-1 isolates are classified intothree groups; M, N and O with M as the main group. The group M is responsible for over 90% of HIV-1 worldwide epidemic. The M groupis further subdivided into 9 recognized phylogenetic subtypes or clades; A-D, F-H, J and K excluding E, which is a CRF(Robertson *et al*., 2000). HIV-2 is restricted to countries in West Africa. It represents 3% of total HIV infection globally. Fortunately it is decreasing in prevalence over time(Eholie and Anglaret, 2006).

HIV isgenetically related to other primate lentivirusesand the subtypesare essentially phylogenetically equidistantgenerating a star like rather than a treelikephylogenyas demonstrated in Figure 1.

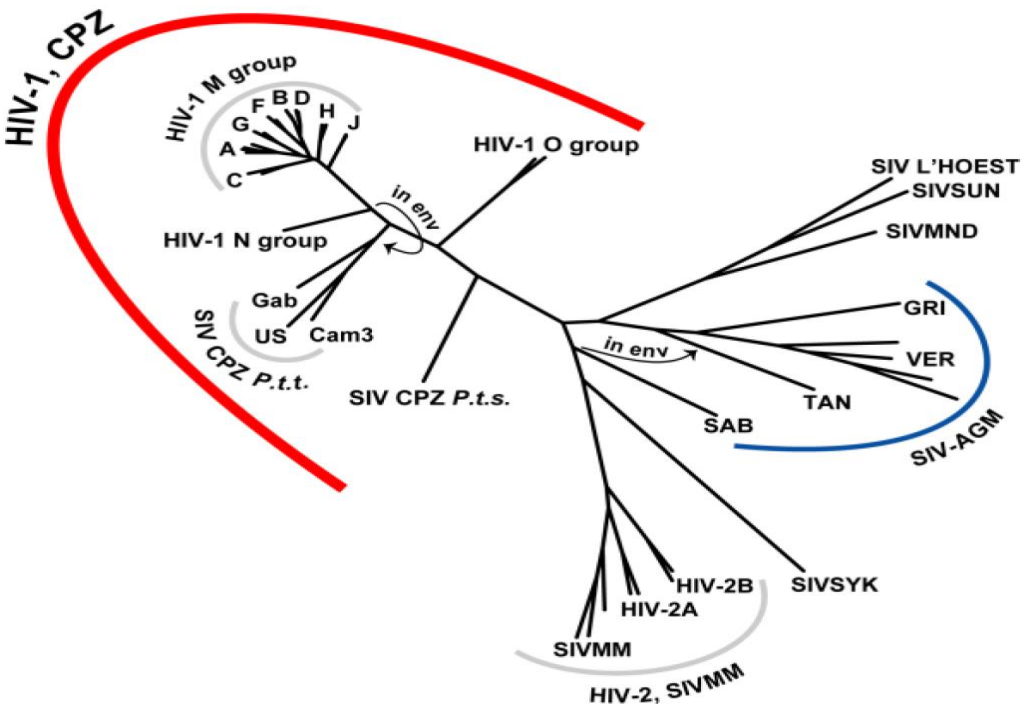


Figure 1: Evolutionary relationships among non-recombinant HIV-1 strains and primate lentiviruses**.**The *pol*gene was used to generate the un-rooted tree. The small arrows in the tree indicate where the sequences would branch in an *env*gene tree. Adopted from Los Alamos National Laboratory, NM).Source: http/www.hiv.lanl.gov(accessed on March2015).

HIV-1 phylogenetic classifications are currently based either on nucleotide sequences derived from multiple sub genomic regions; group antigen (*gag),* polymerase (*pol*)and envelope(*env*) of the same isolates or on full-length genome sequence analysis. This approach has revealed viral isolates in which phylogenetic relations with different subtypes switch along their genomes. The extensive genetic variability of HIV-1 is due to host immune factors, selection pressure, rapid turnover rate, error prone reverse transcriptase that can switch between templates during replicationand recombination of different strains in co-infected individuals (Robertson *et al*., 2000).

When an identical recombinant virus is identified in at least three epidemiologically unlinked people and is characterized by full-length genome sequencing it can be designated as circulating recombinant forms (CRFs). The intra-genomic recombination appears to be a very frequent event

and the CRFs account for 18% of incident infections in the global HIV-1 pandemic (Robertson *et al*., 2000).These inter-subtype recombinant forms are thought to have originated in co-infected individuals, multiply with viruses of two or more subtypes. This results in the generation of several recombinants called unique recombinant forms (URFs)(Peeters and Sharp, 2000). Some CRFs have recombined further with other subtypes or CRFs giving rise to second-generation recombinants (SGRs).

Studies have demonstrated thedifference in HIV-1 clades and subtypesgeographic distribution: subtypes A and D are predominant in Africa; subtype B in USA, Europe, Australia, Thailand and Brazil; subtype C in South Africa, Ethiopia and India; subtype F in some regions of Central Africa and Eastern Europe and CRF01_AE in southeast Asia as shown Figure 2.With the increase in prevalence of HIV, geographic distribution of subtypes has diversified to a large extent. The greatest genetic variation in HIV-1 has been found in regions where HIV epidemic is oldest such as the regions of sub-Saharan Africa where most of the HIV-1 subtypes and many of the CRFs have been identified (McCutchan, 2000).In Kenyarecent studies have demonstrated high genetic diversity with subtype A aspredominant and a high proportion of emerging intersubtype recombinants(Adungo*et al*., 2014; Khamadi*et al*., 2005; Oyaro *et al*., 2011; Yang *et al*., 2004). HIV-1 subtypes circulating in the Northern Kenya town of Moyale mainly comprise subtype A (50%), subtype C (39%) and subtype D (11%).Notablythere is an increase in subtype C in this part of the country. This is probably influenced by neighboring Ethiopia which is dominated by HIV-1 subtype C(Khamadi*et al*., 2005). It has been suggested that this scenario is indicative of cross-border movements influencing circulation of subtypes in Northern Kenya (Khamadi*et al*., 2005). Evidence of increasing HIV-1 diversity is emerging in other parts of the country. Subtypes A1, A2, C, D and CRFsA1/D, A2/D, A1/G, A1/C were identified within the cosmopolitan Nairobi(Lihana*et al*., 2006). Data from Western Kenya along the Kenya- Uganda border shows the emergence of A1/C and A1/D recombinants (Adungo*et al*., 2014)

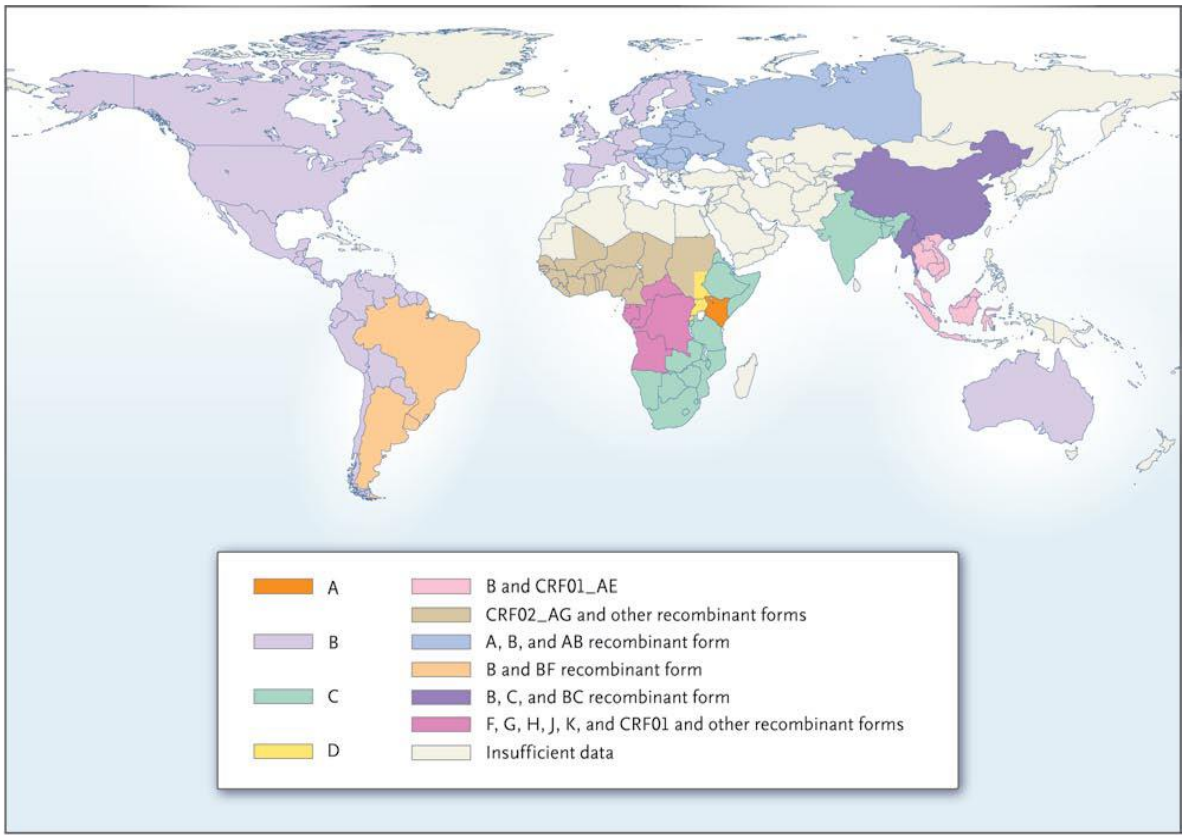


Figure 2: Global Distribution of HIV-1 subtypes and recombinant forms.(Taylor and Hammer, 2008).

2.2 HIV pathogenesis

HIV-1 is a retrovirus belonging to the family of lentiviruses. Like most replication competent retroviruses HIV depends on three genes: *gag*, *pol* and *env*(Wong-Staal, 1991). The classical structural scheme of a retroviral genome is: 5'LTR-*gag*-*pol*-*env*-LTR 3'. The LTR (long terminal repeat) regions represent the two end parts of the viral genome, that are connected to the cellular DNA of the host cell after integration and do not encode for viral proteins. The *gag* and *env* genes code for the nucleocapsid and the glycoproteins of the viral membrane; the pol gene codes for the reverse transcriptase and other enzymes. In addition, HIV-1 contains six genes (*vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*) that contribute to its genetic complexity(Wong-Staal, 1991).

HIV replication occurs mainly within the host CD4+ T-Lymphocytes which are the primary receptors (Dalgleish*et al*., 1984; Klatzmann*et al*., 1984). Experiments showed that expression of human CD4+ T lymphocyte alone on the cell surface was not sufficient to allow entry of HIV.Additional human co-receptors necessary for viral entry wereidentified; the co-receptors are chemokine receptors CCR5 and CXCR4. CCR5 is a necessary co-receptor for monocytotropic (M-tropic) HIV-1 isolates (Deng *et al*., 1996; Doranz*et al*., 1996; Dragic*et al*., 1996). CXCR4 (fusin) was described as the co-receptor used by T-cell-tropic (T-tropic) HIV isolates (Feng*et al*., 1996).

Natural historical cohorts have demonstrated that regardless of populations infected by HIV-1 in all geographical areas, the median time from infection to development of AIDS is approximately 12 years (Weber*et al*., 2001). During the initial phase of HIV-1 infection, there is a burst of virus into the plasma followed by a relative decline in viremia. During this time a strong HIV-1 specific cytotoxic T-cell response is generated which coincides with the early suppression of plasma viremia in most patients.The course of HIV infection has three main phases as illustrated in figure 3; acute phase that occurs after primary infection. It is associated with high plasma viremia and frequently a marked decrease in CD4+ T lymphocytes suggesting a direct relationship between viral replication andCD4+ T lymphocyte destruction.Persistent generalized lymphadenopathy is the common clinical symptom. This is followed by the clinical latency also referred to as asymptomatic phase. During this phase equilibrium between viral replication and host immune response is reached. This chronic stage is also characterized by an active HIV-specific humoral and cellular immune response. Turnover of both CD4+ and CD8+ T lymphocytes is elevated. However in late phase, when the CD4+T lymphocyte count is less than 200cells/µL there is evidence of an increase in the rate of CD4+T lymphocyte decline. Atthis stage a number of AIDS defining illness like wasting syndrome, Tuberculosis, Toxoplasmosis, Kaposi sarcoma occur occasionally leading to death.

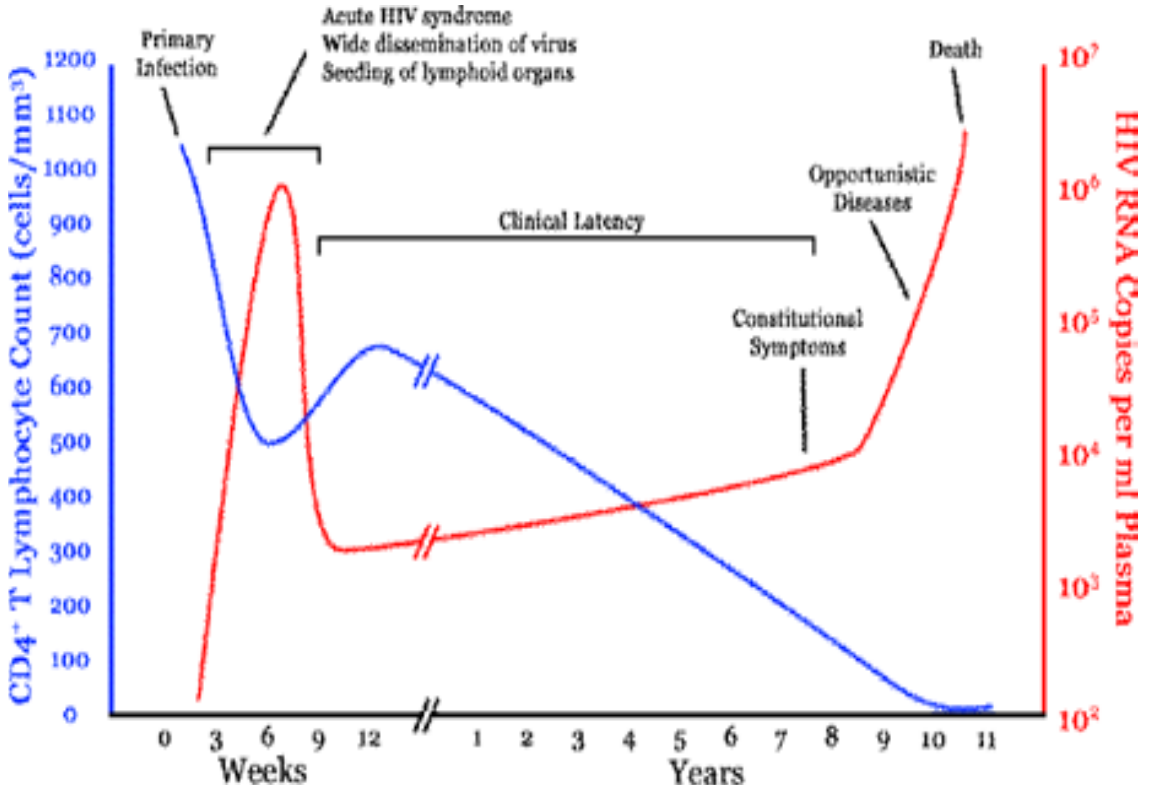


Figure 3: Natural course and immunopathogenesis of HIV(Pantaleo*et al*., 1993)

2.3 HIV prevention and treatment

HIV epidemic has brought extraordinary toll on human life and public health worldwide that can only be reversed with effective prevention and treatment.Numerous prevention interventions exist to combat HIV.Experts recommend that prevention be based on knowledgeof epidemic. This involves tailoring prevention to the local context, epidemiology and using a combination of prevention strategies. Among these prevention strategies include behavioral, structural and biomedicalinterventions(Cohen*et al*., 2005; Piot*et al*., 2008; UNAIDS, 2013a; Weber, 2001).

Behavioral interventions discourage risky behaviors. Instead they reinforce protective behaviors by addressing knowledge, attitudes, skills and beliefs. Unprotected sex is the leading cause of HIV infections accounting for 80% of infections. Therefore comprehensive condom use programs remain an essential component of prevention program in addition to sexual education. Other programs discourage multiple sexual partnerships, early sexual debut and transactional sex(Stoneburner and Low-Beer, 2004).

A structural approach to HIV prevention involves implementing a set of interventions that address factors reducing HIV risk at individual and group level. Structural factors are outside individual knowledge or awareness but have the potential to influence the vulnerability of individuals and groups to HIV infection. Some structural interventions includesocial strategies to eliminate stigma, gender inequality; legal-political strategies like laws and regulations on prostitution; cultural strategies like discouraging wife inheritance and economic empowerment to prevent transactional sex in search of better livelihood(Piot*et al*., 2008)

Biomedical interventions use medical (ARVs) approaches to treat those infected in addition as prevention by blocking infection, decreasing infectiousness and reducing susceptibility. Combination ARVfirst introduced in 1996, has led to dramatic reductions in morbidity and mortality. There are five different classes of ARV: Nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Entry inhibitors and Integrase inhibitors. NRTIs contain modified ribose molecules which compete with physiological nucleosides to bind at the reverse transcriptase active sites. This inhibits formation of phosphodiester bonds hence blocking HIV-1 DNA synthesis. NNRTIs unlike NRTIs do not require activation in the cell and non-competitively bind to the reverse transcriptase non active sites. This result in blocking the catalyst activated binding sites slowing HIV-1 DNA polymerization significantly (Andries*et al*., 2004). Protease inhibitors act by inhibiting HIV-1 protease from splicing *gag-pol* polyprotein into functional subunits, this result in non-infectious virus (Youle*et al*., 2007). To inhibit the HIV-1 from entering the CD4+ T lymphocyte, Entry inhibitors act as co-receptor antagonists of fusion inhibitors (Moore and Doms, 2003). To block the integration of HIV-1 DNA into the host genome, Integrase inhibitors bind to the Integrase active sites preventing replication of the virus (Nair *et al*., 2002). Effective ART reduces VL lowering the risk of HIV transmission. Patients with repeated episodes of detectable viremia suggesting ongoing viral replication rather than viral release from latent reservoirs due to immune activation are at increased risk of developing drug resistance. The highest probability of drug resistance to any drug class is between 1000 and 10,000 copies/ml (Prosperi*et al*., 2011). Development of HIV-1 resistance depends on the selection of resistance-associated mutations (RAMs). If a virus has acquired one or more RAMs leading to reduced drug sensitivity, the mutant virus attains a replication advantage in comparison to wild-type virus

when exposed to ART (Drake *et al*., 1993). The development of resistant viral strains is one of the main reasons for virological failure in ART.In addition ARV has been used for pre-exposure prophylaxis, post-exposure prophylaxis and microbicides to prevent HIV infection. Among other biomedical interventions include prevention of mother to child transmission, voluntary HIV testing, blood supply safety andvoluntary male circumcision. HIV vaccine research is also ongoing and will contribute to this strategy(Padian*et al*., 2008; UNAIDS, 2014b).

2.4 HIV-1 diagnostic and viral load testing technologies

Among the key diagnostic tests required for HIV patients include immunological staging and continuous monitoring of patients on ART. Accessibility of immunological staging tests using CD4+T lymphocyte counts for initiating treatment is universal. Similarly, uptake and accessibility of serological testing for anti-HIV antibodies in adults has improved significantly due to the rapid POC tests. The remaining challenge especially in developing countries is accessibility of EID and VL monitoring which require sophisticated molecular tests.

2.4.1 Early Infant Diagnosis

Infants mainly acquire HIV through mother-to-child transmissionduring pregnancy, at birth and through breast milk. Exposed infants below 18 months do have persistent maternal antibodies limiting the use of antibody tests due to possible false positive diagnosis. Instead virological testing (either RNA PCR or DNA PCR testing) or ultrasensitive p24 antigen testing is recommended tobe used to diagnose infants in this age group. Current WHO guidelines call for all HIV-exposed infants to have virological testing at 4 to 6 weeks of age or atthe earliest opportunity thereafter (WHO, 2013). The qualitative HIV-1 DNA test detects the presence of HIV proviral DNA. It provides a “yes” (positive) or “no” (negative)result with respect to whether the infant is infected with the HIV virus. Considerable uptake of EID has been seen though access is far from universal availability(WHO, 2013).

2.4.2 Viral load testing

Viral load is quantification of circulating HIV-1 RNA in blood. It is the preferred method for monitoring HIV patients once they have been initiated onto ART. High levels of HIV circulating in the bloodstream indicate that the virus is actively replicating.These levels can be used with the aid of molecular methods to provide important informationregarding the risk of disease

progression, predict ARToutcome, assess infectivity and monitor emergence of viral resistance.(Constantine *et al*., 2005).ART initiation interrupts viral replication resulting in decreased level of viral particles in the host. This slows the progression of the disease and improves the patient's prognosis. Therefore patient's VL levels can be used as an indicator of the efficiency of therapy along with clinical symptoms and CD4+ T lymphocyte counts. VL testing is used to determine whether the virus is "undetectable" in the patient's blood (below the limit of detection of currently available technologies as measured in copies of the virus per millimeter). Viral copies below 1000 copies/ml is considered an indicator of viral suppression and successful ART, (WHO, 2013).With the scale up and increased access to ART, the success of treating HIV patients depends on accurate virological monitoring. In addition Kenya has adopted WHO recommendation of routine VL testing (every 6 – 12) for all patientsrather thanthe previous targeted approach for patients who only meet clinical and immunological criteria for the test(WHO, 2013).This has led to scaling of VL testing consequently a rise in patients' numbers requiring the service despite the limited testing capacity.

2.4.3 Current and developing technologies for EID and viral load

Among current existing commercially available FDA approved nucleic acid based tests for HIV-1 EID and VL testing include nucleic acid testing (RT- PCR) technology CAP/CTM and Abbott m2000. CAP/CTM primers targetthe conservedregion of*gag* p41 gene and uses TaqMan differential fluorescence tagged primers to amplify HIV-1 RNA. Additional fluorescence primers and probes detect quantification standards (internal control) and partial sequence of the long terminal repeat (LTR). This assay reliably detects HIV-1 group M; A–D, F–H viruses and several CRFs including CRF01-AE(Roche, 2008). Abbott m2000 primers target the conserved region of *pol-integrase* genes and amplify HIV-1 RNA using a partially double-stranded qRT-PCR method. This assay reliably detects HIV-1 group M; A–D, F– H, several CRFs including CRF01-AE and CRF02-AG as well as group N and O(Abbott, 2007). These technologies are fully automated, sophisticated and utilized in established centralized laboratories.

2.4.4 Challenges in EID and viral load testing

2.4.4.1 Logistical challenges

Access to HIV prevention and treatment has improved significantly over the past years. Nevertheless, major gaps remain between need and access to diagnostic tests in resource-limited settings. The existing global market for HIV diagnostics does not meet the needs of the 34 million people living with HIV(UNAIDS, 2014b). The sophisticated technologies that are dominating the market are ill suited to most resource-limited settings as they are expensive, require complex laboratory infrastructure, stable electricity supply, highly trained laboratory technicians and needs regular maintenance by skilled technicians. The existing centralized laboratories are also operating above capacity(Haleyur*et al*., 2014). Consequently, even with growing demand for EID and VLtestingaccess remains low and therefore laboratory-based tests regarded as standard in developed countries are cost prohibitive and inaccessible to most patients in many developing countries(Mabey*et al*., 2004; Peeling and Mabey, 2010). Recognizing this disparity, WHO has called for new HIV clinical diagnostic technologiesthat can function in settings with restricted access to a central laboratory(Mabey*et al*., 2001).

2.4.4.2 HIV-1 Diversity

Genetic heterogeneity of HIV-1 complicates molecular diagnostics. Ideal assays should be able to detect and quantify all known HIV-I subtypes, recombinant forms and emerging variants. But currently that is not the case although most assays can recognize most but not all variant HIV-1 forms (Swanson *et al*., 2005; von Truchsess*et al*., 2006).In addition, majorityof HIV-1 molecular diagnostics assays have been developed and optimized for subtype Bthat is only dominant in developed countries. When such assays are used in different populations with multiple non-B HIV subtypes, reliable detection and quantification can be compromisedby natural polymorphisms occurring in primer/probe sequences that have the potential to reduce or abolish hybridization(Church *et al*., 2011; Murphy *et al*., 2000). Several comparativestudies using clinical specimens from regions with minimal subtype B prevalence have shown discordant results between assays confirming impact of genetic variability on HIV test performance. In addition,performance has been shown to significantly vary between HIV VL assays depending on HIV-1 subtype. This highlights the significance ofHIV-1 viral diversity prevalencein

choosing HIV-1 diagnostic and monitoring assays(Bourlet*et al., 2011;* Church *et al*., 2011; Murphy *et al*., 2000).

2.4.5 POC technologies

POC test can be defined as a diagnostic test that is performed near the patient or treatment facility, has a fast turnaround time and may lead to a change in patient management(Laursen*et al*., 2012).Therefore the aim of diagnostic POC is to minimize the time to obtain a test resultthereby allowing clinicians to make quick decisions. POC testinghas been available for several years for infectious diseases. For example the rapid HIV antibody–based tests which have been confirmed by over a decade of efficacy and acceptability studies to increase access to HIV diagnosis compared to centralized testing (Appiah*et al*., 2009; Rahangdale*et al*., 2008). Considerable efforts have been spent in developing POC devices including HIV-1 nucleic acid detection, CD4+ T lymphocyte quantitation and VLmonitoring targeting use in resource-limited settings (Blyth *et al*., 2011). Currentlythere are no FDA-approved POC EID and VL nucleic acid tests.

POC technologies currently under development include: PanNat Diagnostic platform and ultrasensitive p24 antigen assayfor EID.Assays for VLtesting include; AlereInc, Liatanalyser and EOSCAPE HIV™.(Haleyur*et al*., 2014).Specific focus of this study isSAMBA developed by Cambridge University. SAMBA is among the first HIV-1 POC nucleic acid based tests in Kenya able to perform both EID and VL assays.The SAMBA HIV-1 test has the potential for HIV-1 nucleic acid signal amplification and rapid visual detection technology for nucleic acid, this makes it suitable for POC applications (Lee *et al*., 2010). In contrast to conventional NATs, the SAMBA test relies on Helicase- Amplification that is an isothermal amplification technology. This technology allows the SAMBA to operate at a single temperature eliminating the need for a thermocycler enabling assays to be conducted on simple and portable heating systems(Chow *et al*., 2008).SAMBA hasa set of primers targeting the conserved region of long terminal.It has capacity for visual readoutof results utilizing vertical-flow DNA detection strip that has an internal control line to validate the proper performance of the reactions. SAMBAdoes not require expensive or complex instrumentation and is thereforeideal for routine use in resource-poor settings(Dineva*et al*., 2005; Lee*et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

This was a laboratory-based study. Specimens were obtained from patient support health facilities within 6 counties; Kisumu, Siaya, Kisii, Migori, Nyamira and Homabay.

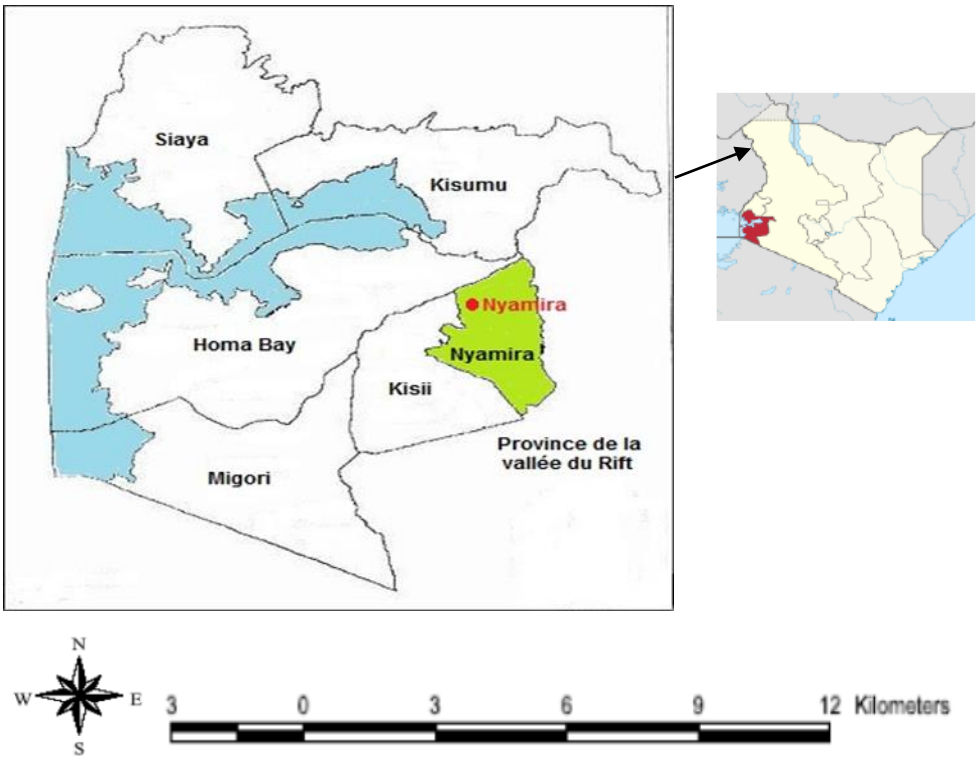


Figure 4: Map of the six counties in Western Kenya.

3.2 Study Design

This was a cross sectional study carried out from March 2014 to February 2015 evaluating the performance of SAMBA-Q and SAMBA-SQ assays in comparison to thereferenceCAP/CTM assay.Patients were recruited at the patient support health facilities where they sought routine clinical care utilizing the existing Ministry of Health trained health care workers and phlebotomists who drew blood specimen. For SAMBA-Q evaluation 112 infants were recruited from Kisumu, 57 from Siaya, 40 from Kisii, 41 from Migori 38 from Nyamira and 47 from Homa-Bay County. For SAMBA-SQ evaluation 59 adults were recruited from Kisumu, 41 from Siaya, 30 from Kisii, 18 from Migori 20 from Nyamira and 32 from Homa-Bay County.

3.3 Study population

Target population included both male and female. For EID,HIV exposed infants below 18 months of age were recruited. For VL test, HIV positive adults on ART seeking care at differentpatient support health facilities were recruited.

3.4 Ethical approval

Ethical approval for the study was obtained from Kenya Medical Research Institute Ethical Review Committee (ERC) and Scientific Steering Committee (SSC). Protocol number SSC 2496.

3.5 Inclusion and Exclusion Criteria

3.5.1 Inclusion criteria

Participants recruited in the study were consented HIV infected adults aged 18 years and above. Additionally infants aged 18 months and below whose parent(s)/guardian(s) provided informed consents were recruited.

3.5.2 Exclusion criteria

Children aged above 18 months born to HIV-infected mothers and infantswho were not born to HIV-1 infected mothers were excluded. While HIV uninfected adults were excluded from the quantitative assays.In addition patients who did not give an assent/consent were not recruited.

3.6 Sample sizedetermination

Sample size was estimated using the Buderer's formula (Buderer*et al*., 1996) to determine theSensitivity(S_N) of 96.99% for SAMBA EID and 98.0% for SAMBA VL with absolute precision of less than 5% ata 95% confidence interval [CI] with a 15.1% prevalence of HIV in former Nyanza province(KAIS, 2014).

$$n\text{based on sensitivity} = \frac{Z^2_{1-\alpha/2} \times S_N \times (1 - S_N)}{L^2 \times \text{Prevalence}}$$

Where n = required sample size

S_N= anticipated sensitivity

α = size of the critical region ($1 - \alpha$ is the confidence level)

$Z_{1-\alpha/2}$= standard normal deviate corresponding to the specified size of the critical region (α)=1.96

L = absolute precision desired on either side (half-width of the confidence interval) of sensitivity

$$n\text{forSAMBAEID} = \frac{1.96^2 \times 0.9699 \times (1-0.9699)}{0.05^2 \times 0.151} = 297.09$$

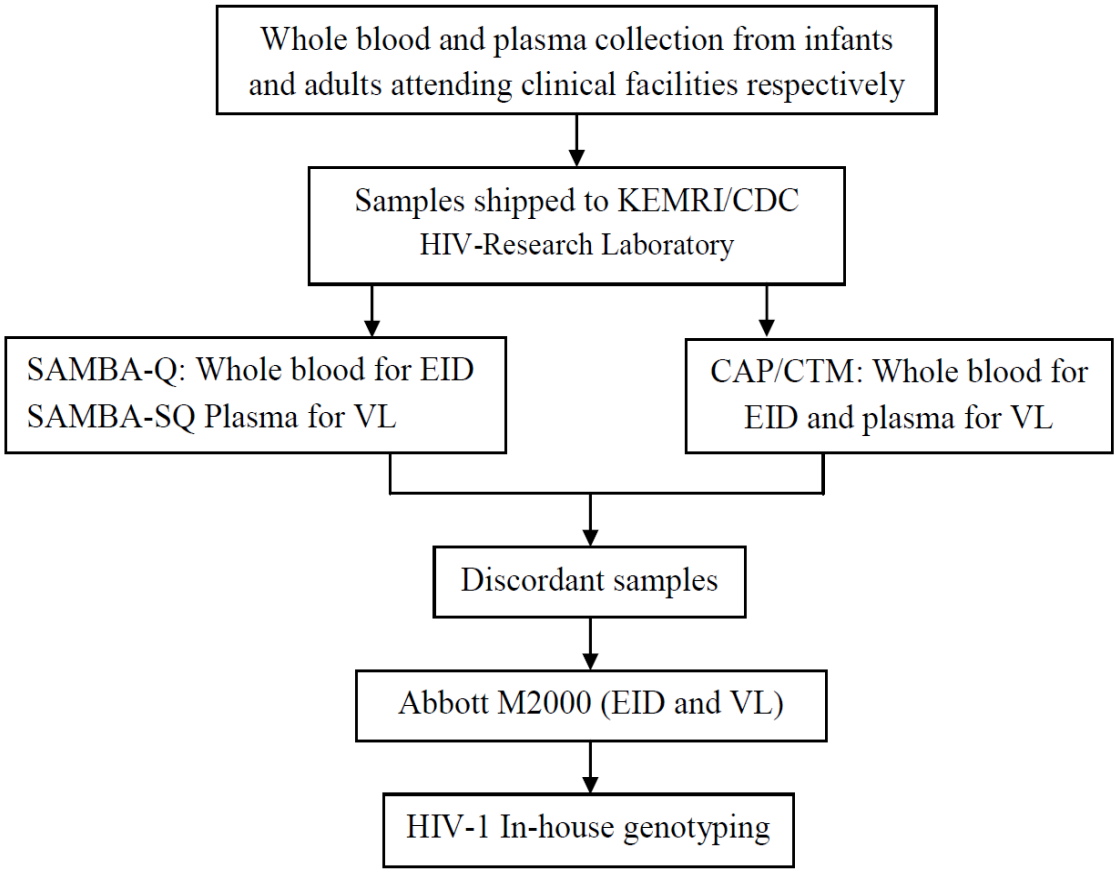
$$n\text{forSAMBAVL} = \frac{1.96^2 \times 0.98 \times (1-0.98)}{0.05^2 \times 0.151} = 199.45$$

Based on the above formula the minimum sample size (n) was 297and 200 for EID and VL respectively.

3.7 Sampling and specimens collection

For SAMBA-Q assay evaluation, 335 infants (200 HIV-positive and 135 HIV-negative) were recruitedrandomly from the six counties. To evaluate performance of SAMBA-SQ test, 200 HIV-1 positive adults were recruited based on the following reference CAP/CTMVL categories: undetectable, < 1000 copies/ml, 1000 copies/ml – 9999 copies/ml, 10,000 copies/ml – 99,999 copies/ml and >= 100,000 copies/ml with a sample size of 50 in each group.

Specimens were collected from patients following consenting /assenting. For EID,1 ml of venous whole blood was collected into EDTA vacuitainer tubesfrom infants aged 18 months and below born to HIV infected mothers. Similarly for VL monitoring, 4ml whole blood sample was collected intoEDTA vacuitainer tubes from HIV infected patients requiring clinical management from the ministry of health facilities. Whole blood from infants was shipped to the testing laboratory in ice packs.Whole blood from the adults was separated at the health facilities by centrifugation to obtain plasma thatwas then frozen and shipped to the testing laboratory. Once in the laboratory specimen were aliquoted for storage at -80°C prior to testing to minimize HIV-1 nucleic acid degradation by freeze-thaws.



Flow chart 1:EID (whole blood) collected from infants and VL (plasma) collected from adults were performed on both SAMBA (SAMBA-Q and SAMBA-SQ) and reference standard of care CAP/CTM. Concordant results were reported. Discordant results were tie-broken using Abbott(primers targeting *pol-int* HIV gene) to obtain definitive results. Since it was hypothesized that discordance may be related to genetic diversity, any discordant specimens were further genotyped to determine the infecting HIV-1 subtype.

3.8 Experimental procedures

To diagnose HIV-1 in infants, pro-viral DNA was extracted from whole blood.To quantify HIV-1VL in plasma, non-cell associated RNA was extracted. Both nucleic acid extracts were amplified and detected as described below.

3.8.1 HIV-1 Pro-viral DNA extraction for Early Infant Diagnosis

UsingCAP/CTM 48 analyzer (Roche Diagnostics, Ltd., Rotkruez, Switzerland), pro-viral DNA extraction, amplification and detectionwereperformedfollowing the manufacturer's standard guidelines.Briefly, 100µL of each whole blood specimen was transferred into a sample tube (S-tube) containing 1000 µLof sample pre-extraction (SPEX) buffer and incubated at 56^0C for 10 minutes with intermittent shaking. After incubation, the S-tubes were placed on CAP for pro-viral DNA extraction and eventual amplification using RT- PCR technology for detection in CTM. Results were read as either HIV-1 detected or not detected (Positive or Negative)(Appendix 6).

SAMBA-Q reagentswere used to extract pro-viral DNA from 100 µL of whole blood on the specimen-preparation equipment(SAMBAprep). The nucleic extracts were transferred into the SAMBAamplification (SAMBAamp) equipment andisothermal amplification performed according to the manufacturer's guidelines. The results were read visually onSAMBAampcartridge dipstick (Figure 5 and 6). The presence of a control line indicated a valid test and the test line on the dipstick indicated Pro-viral DNA detected, whereas the absence of the test line indicated Pro-viral DNA not detected. The absence of both lines indicated an invalid test result.

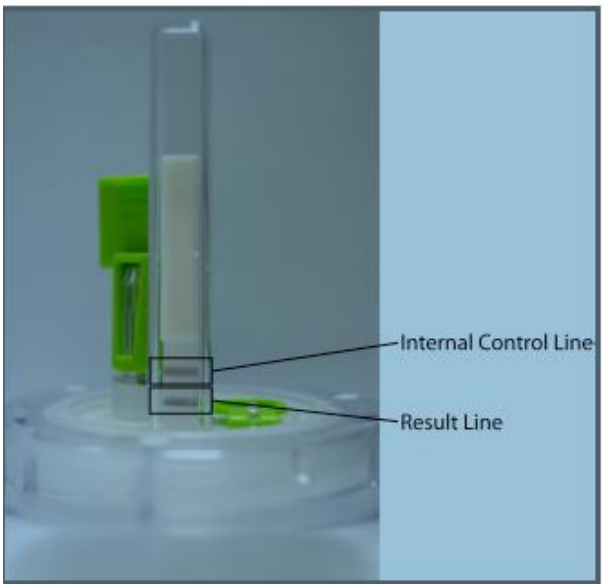


Figure 5: Visual detection of nucleic acid by dipstick in the SAMBA cartridge using the signal amplification system. Internal control line is for quality assurance.

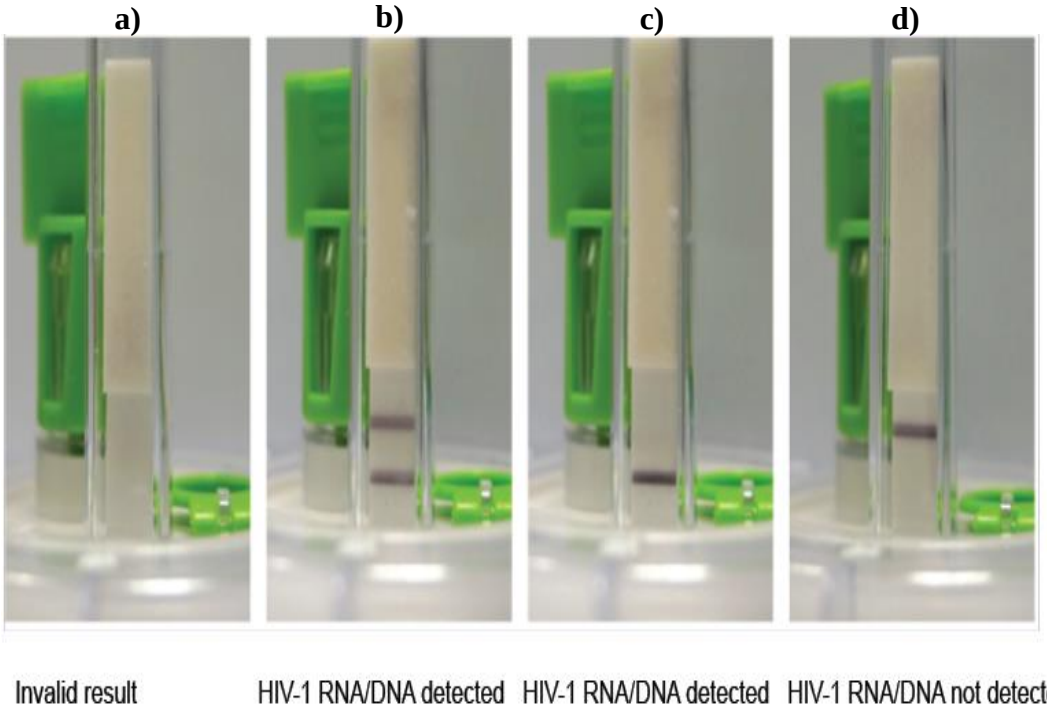


Figure 6: SAMBA-Q amplification cartridges showing EID results interpretation. a)Invalid, b) HIV-1RNA/DNA detected, c) HIV-1RNA/DNA detected (common in specimen with HIV RNA/DNA above 10^6copies/ml outcompeting the control, hence control signal is very weak or not visible)and d) HIV-1RNA/DNA not detected.

Using Abbott m2000sp/Abbott m2000rt analyzer (Promega, Madison, WI, USA), pro-viral DNA extraction, amplification, and detection were performed following the manufacturer's standard guidelines; 100 µL of each whole blood specimen was transferred into a reaction vessel containing 1000 µL of bulk lysis and incubated at room temperature for 10 minutes with intermittent shaking. After incubation, the reaction vessels were placed in Abbott m2000sp for pro-viral DNA extraction and eventual amplification using RT- PCR technology for detection in Abbott m2000rt analyzer. Results were read as HIV-1 detected or not detected (Positive or Negative)(Appendix 7).

3.8.2 HIV-1 RNA extraction and quantitation for viral load testing

Using CobasAmpliprep/CobasTaqMan 48 analyzer (Roche Diagnostics, Ltd., Rotkruez, Switzerland) circulating RNA extraction, amplification and quantification was done following the Roche manufacturer's standard guidelines. For each plasma specimen, 1100 μL was aliquoted into the respective S-tubes after vortexing for 5 seconds. The S-tubes were placed on CAP for extraction of circulating RNA and eventual amplification using RT- PCR technology for quantification in CTM. Results were read as HIV-1 quantified (20 -10,000,000copies/ml) or not quantified (below 20 copies/ml)(Appendix 6).

SAMBA-SQ reagents wereused to extract HIV-1 RNA from 200 μL of plasma on the SAMBAprep.The nucleic extracts were transferred into the SAMBAamp equipment and isothermal amplification preformed according to the manufacturer's standard guidelines. The results were read visually onSAMBAampcartridge dipstick (Figure 6). The presence of a control line indicated a valid test and the test line on the dipstick indicated a VL of ≥1000 copies/ml. The absence of the test line indicated VL <1000copies/ml. The absence of both lines indicated an invalid test result (Figure 6).

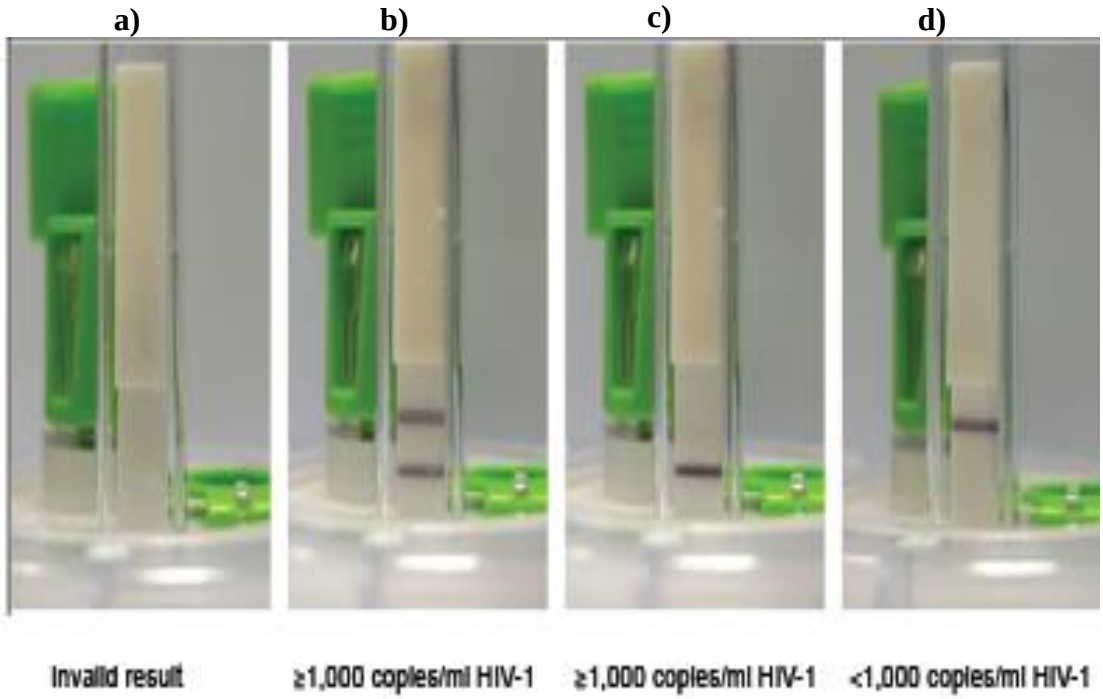


Figure 7: SAMBA-SQ amplification cartridges showing VL results interpretation.a) Invalid, b) ≥ 1000 copies/ml, c) ≥ 1000 copies/ml(common in specimen with HIV RNA/DNA above 10^6copies/ml outcompeting the control,hence control signal is very weak or not visible)and d) < 1000 copies/ml.

UsingAbbott m2000sp/Abbott m2000rt analyzer (Promega, Madison, WI, USA),HIV-1RNA extraction, amplification, and detectionwere performedfollowing the manufacturer's standard guidelines; 600 µL of each plasma specimenwas transferred into a reaction vessel. The reaction vesselswere placed in Abbott m2000sp for RNA extraction and eventual amplification and quantification using the RT- PCR technology in Abbott m2000rt analyzer. Results were read as HIV-1 quantified(40 -10,000,000copies/ml) or not quantified (below 20 copies/ml) (Appendix 7).

3.8.3 HIV-1 Genotyping

An in-house assay approved by WHO for genotyping of HIV-1 *pol* region (protease and partial reverse transcriptase genes) was used for genotyping of discordant specimens (i.e. specimens whose results between SAMBA and CAP/CTM did not match). *Pol* gene is variable with mutations conferring resistance commonly occurring at this region making a suitable alternative for HIV-1 subtyping(Zhou *et al*., 2009). The assay amplifies 1,084-bp fragment of the HIV-1 *pol*

gene encoding amino acids 6 to 99 of the protease region and codons 1 to 251 of the reverse transcriptase (RT) region. (Inzaule*et al.*, 2013; Zhou *et al*., 2009).The procedure used for HIV-1 RNA extraction, amplification (RT-PCR and Nested PCR) and sequencing are as described below.

3.8.3.1 Extraction of HIV-1 viral RNA

Was performed using the QIAGEN QIAamp Viral RNA Mini Kit (QIAGEN, Valencia CA) according to the manufactures' guideline. Briefly, 140 µLeach plasma/whole bloodwas added to 560 µL of buffer AVL containing carrier RNA in a microcentrifuge tube. Buffer AVL contains a chaotropic salt guanidine thiocynate facilitating cell lysis to release the nucleic acids. Following incubation at room temperature (18-25^{0}C) for 10 minutes, 560 µL of ethanol was added and mixture vortexed briefly. Two consecutive aliquots of 630 µLwere added to the QIAamp spin column and centrifuged at 8,000 revolutions/min−1 (rpm) for 1 min. The QIAamp spin column was thentransferred into a clean 2 mL collection tube followed by addition of 500 µL of Buffer AW1to wash the pellets and centrifugation done as described above. This was followed by addition of 500 µL of Buffer AW2 for a second wash and centrifuged at 16,400 rpm for 3 min. The QIAamp spin column was then transferred to 2ml collection tube and centrifuged at 16,400 rpm for 1 minute to eliminate any Buffer AW2 carry over that may interfere with the PCR. The QIAamp spin column was then placed in a clean 1.5 mL microcentrifuge and incubated with 80 µL of buffer AVEeluent for 1 min.HIV-1 RNA wereobtained by final centrifugation step at 8,000 rpm for 1 min.

3.8.3.2 RT-PCR

To generate clones of HIV-1 complementary DNA (cDNA), outer primers PrtM-F1 / PrtM2-F1(forward, 5'-CCT CAA ATC ACT CTT TGG CAR CG, nucleotides [nt] 2253 to 2275 based on HXBII) and RT-R1(reverse, 5'-ATCCCT GCA TAA ATC TGA CTT GC, nt 3370 to 3348) (Zhou *et al*., 2009)were utilized.The final 50 µLPCR mixture contained 2 X Reaction Mix, 10µM PrtM-F1 / PrtM2-F1, 10 µM RT-R1, DEPC-treated Water, Invitrogen Super Script III one-step RT-PCR High Fidelity enzymeand 15 µL of the extracted RNA. Thermocycling conditions were as follows: RNA denaturation at 65^{0}C for 10 minutes, annealing and

amplification at 50^0C for 45 minutes to obtain cDNA. This was followed by denaturation of cDNA at 94^0C for 2 minutes, 40 cycles of denaturation at 94^0C for 15 seconds, annealing at 50^0C for 20 seconds and extension at 72^0C for 2 minutes.

3.8.3.3 Nested PCR for the pol gene

PCR amplification of the entire of HIV-1 *pol*gene was performed with two sets of inner primers Prt-F2(forward, 5'-CTT TGG CAA CGA CCC CTY GTC WCA, nt 2265 to 2288), RT-R2(reverse, 5'-CTT CTG TAT GTC ATT GAC AGTCC, nt 3326 to 3304)(Zhou *et al*., 2009)and Taq polymerase (Applied Biosystems (ABI)California USA).The PCR reaction mixture of 50 µL contained DEPC Treated Water, 10x PCR Buffer, 25mM $MgCl_2$, 10mM dNTPs, 10µM Prt-F2, 10µM Prt-F2, Taq polymerase and 2 µL of the cDNA products.Thermocycling conditions were as follows: denaturation at 94^0C for 4 minutes, followed by 40 cycles of denaturation at 94^0C for 15 seconds, annealing at 55^0C for 20 seconds and extension at 72^0C for 2 minutes.The amplified products were electrophoresed on 1 % agarose gelstained by SYBRgreen dye at 10V/cm and visualized under ultraviolet light (Appendix 8).

3.8.3.4 DNA sequencing

Nested PCR products of *pol* gene were partially sequenced using the BigDye Terminator method (Applied Biosystems, California USA). Six sequencing primers overlapping the entire amplicon were used to generate a *pol* gene fragment spanning the protease (amino acid 6-99) and the reverse transcriptase (amino acids 1-251)using sequencing primers A35V (forward, 5'-AGT CCT ATT GAR ACT GTR CCA G, nt 2556 to 2577, AV36V (forward, 5'-CAG TAC TGG ATG TGG GRG AYG, nt 2869 to 2889 , AV44 (reverse, 5'-TTT YTC TTC TGT CAA TGG CCA, nt 2639 to 2619, 90V1 (reverse, 5'-TAC TAG GTA TGG TAA ATG CAG T, nt 2952 to 2931, PRT-F2(forward, 5'-CTT TGG CAA CGA CCC CTY GTC WCA, nt 2265 to 2288) and RT-R2 (reverse, 5'-CTT CTG TAT GTC ATT GAC AGT CC, nt 3326 to 3304) (Zhou *et al*., 2009). The 20 µL sequencing reaction mixture contained Big dye terminator, Big dye 5x Buffer, dH_20, 10 µM Primer and 8 µL Nested PCR products.Thermocycling conditions were as

follows:25 cycles of denaturation at 96^0C for 10 seconds, annealing at 50^0C for 5 seconds and extension at 60^0C for 4 minutes.The products were then purified by transferring 20 µL of Dye Deoxy TM terminator reaction mixture to the top at centre of the gel bed top of the of a well centricep gel column. The column was placed into a labeled 1.5 mL sample collection tubes and transferred into the rotor with the highest point of the gel media in the column always pointing to the outside of the rotor. This assembly was the centrifuged at 750 xg (3000 rpm) for 2 minutes. The purified big dye sequence products collected at the bottom of the1.5ml sample collection tubes. The sequenced products were then analyzed in ABI 3130xl Genetic Analyzer (Applied Biosystems).

3.9 Data Management and Analysis

3.9.1 Statistical analysis

Data obtained from SAMBA, CAP/CTM and Abbott was entered in computer software MS Excel 2007. Sequences were saved in FASTA format as text.The data in MS Excel were then transferred to STATA version 13 software (Stata Corp, Inc., College Station, Texas, USA) for analysis. Accuracy (bias and limits of agreement) of the POC SAMBA device in comparison to CAP/CTMwas calculated for the binary VL (<1,000 versus => 1,000 copies/ml) and EID (yes or no) variables. Sensitivity, specificity, positive and negative predictive estimateswere alsocalculated for the POC SAMBA test with respect to CAP/CTM with values ≥70% defined as acceptable.Concordance correlation and agreement between the 2 assays was assessed with Cohen Kappa Agreement was interpreted as weak ($0.4 > k \geq 0.2$), moderate ($0.6> k \geq 0.4$), strong ($0.8> k \geq 0.6$), nearly perfect ($1.0 > k \geq 0.8$) and perfect ($k=1.0$).Exact 95% confidence intervals were calculated for all estimates with $p<0{\cdot}05$ being considered statistically significant.

3.9.2 Phylogenetic analysis

The obtained HIV-1 sequences in FASTA format were assembled and edited using stand-alone RECall version 2.2 software(University of British Columbia, USA). To determine HIV-I subtypes, PCR contamination was ruled out and sequence quality checked using Sequence Quality Assessment Tool (SQUAT). Thederivedsequences were aligned along selected reference sequences representing relevant HIV-1 subtypes and circulating recombinant forms (CRFs) from

the GenBank database using CLUSTALW multiple alignment program(Thompson *et al*., 1994). After manual adjustments and stripping gap sites using BioEdit(Hall, 1999). Phylogenetic treewas constructed by the neighbour-joining method using MEGA version 6.0 software. The reliability of the branching nodes was assessed by the bootstrap analysis using 1000 replicates. Boot- strap values ≥ 70% were considered significant (Hill and Bull, 1993).Genetic distances were calculated with the two-parameter method of Kimura(Tamura *et al*., 2013).

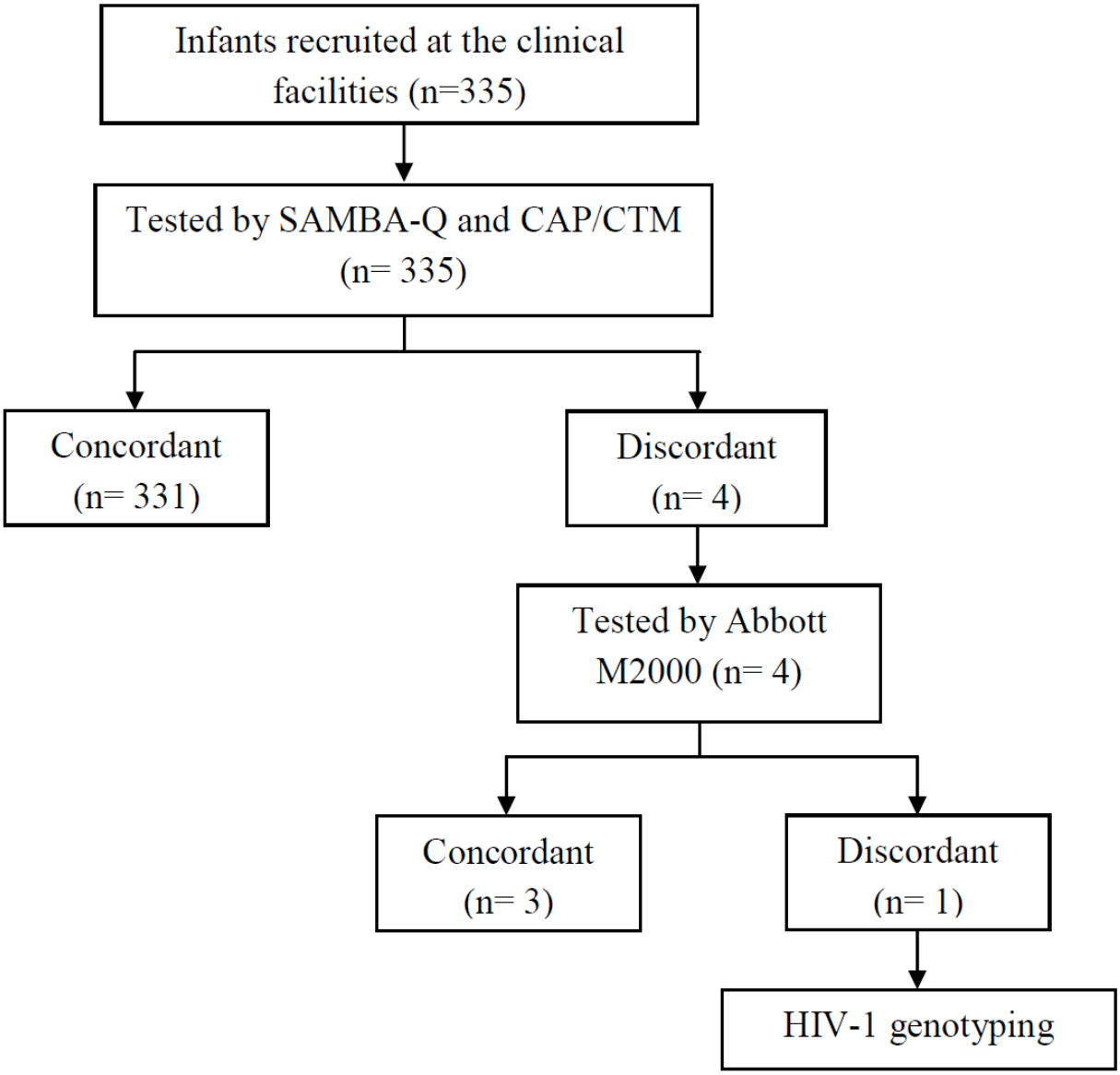
CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1 Results

4.1.1 SAMBA-Q Evaluation

As summarized in flow chart 2; following screening using CAP/CTM,335HIV-1 exposed infants were recruited for SAMBA-Q evaluation. Among these, 200 were HIV-1 positive and 135 HIV-1 negative. Aliquotedwhole blood specimenscollected from each infant were tested on SAMBA-Q and CAP/CTM platforms. Of these, 200 were concordantly positive while 131 were concordantly negative. Four specimens were discordant between the two platforms: 3 negative and 1 positive on SAMBA-Q.To resolve the 4 discordant results;the specimens were tested on the Abbott m2000 assayas a tie-breaker.The 3 specimens that were negative on SAMBA-Q remained negative on the Abbott m2000while the positive turned negative on the same assayand was genotyped.



Flow chart 2: Algorithm used for testing infants' whole blood specimensfor HIV-1 diagnosis.Three hundred and thirty five specimens were tested by SAMBA-Q and CAP/CTM. 4 discordant specimens were tie-broken by Abbott and one that remained discordant was genotyped to determine HIV-1 subtypes.

SAMBA had a sensitivity of 98.5% (95% CI: 95.7-99.7), specificity of 99.2% (95% CI: 95.9-100), positive predictive value of 99.5% (95% CI: 97.3-100), negative predictive value of 97.8% (95% CI: 93.6-99.5) and an overall concordanceof 98.8% (95% CI: 98.2-99.5) when compared to reference CAP/CTM (Table 1).

Table 1: Comparison of SAMBA-Q test performance against the CAP/CTM(*n*=335) assessing sensitivity, specificity, PPV and NPV.

HIV test	**CAP/CTM+***	**CAP/CTM-****	**Total**
SAMBA +	200	1	201
SAMBA -	3	131	134
Total	203	132	335
Sensitivity	98.5 (95.7-99.7)		
Specificity	99.2 (95.9-100)		
PPV	99.5 (97.3-100)		
NPV	97.8 (93.6-99.5)		
Concordance	98.8 (98.2-99.5)		

***+**: *Positive*

**-: *Negative*

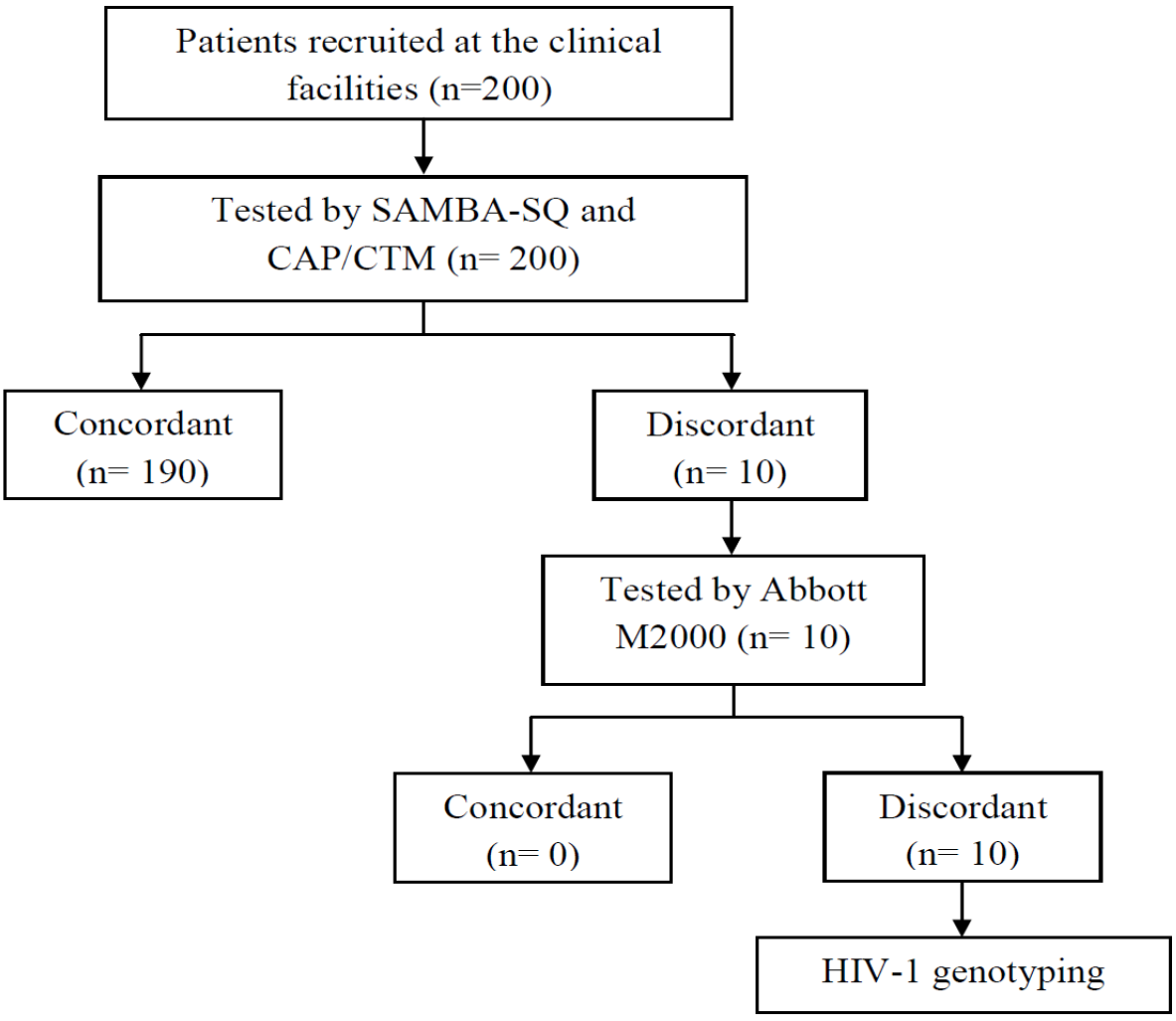
After tie-breaking the 4 discordant results with Abbott,the resolved SAMBA-Q sensitivity was 100% (95% CI: 98.2-100), specificity of 99.3% (95% CI: 95.9-99.9), positive predictive value of 99.5% (95% CI: 97.3-100), negative predictive value of 100% (95% CI: 93.6-100) and an overall concordanceof 99.7% (95% CI: 99.1-100) with a Cohen Kappa value of 0.99 (95% CI: 0.98-1.00) (Table 2).

Table 2: Comparison of SAMBA-Q test performance with combined CAPCTM and Abbott (*n*=335) assessing sensitivity, specificity, PPV, NPV and agreement using Cohen Kappa analysis followingAbbott tie-breaking of discordant results.

HIV test	**CAP/CTM+**	**CAP/CTM-**	**Total**
SAMBA +	200	1	201
SAMBA -	0	134	134
Total	200	135	335
Sensitivity	100 (98.2-100)		
Specificity	99.3 (95.9-100)		
PPV	99.5 (97.3-100)		
NPV	100 (93.6-100)		
Concordance	99.7 (99.1-100)		
Cohen Kappa	0.99 (0.98-1.00)		

4.1.2 SAMBA-SQ Evaluation

Two hundred patients on ART were recruited for the SAMBA-SQ evaluation.As summarized in flow chart 3,aliquoted plasma specimens from each adult patientwere tested on SAMBA-SQ and CAP/CTM platforms.One hundred and ninety were concordant while 10 were discordant. The discordant specimens remained so even after tie-breaking with Abbott and were genotyped.



Flow chart 3:Algorithm used for testing adults' plasma for viral load quantitation using SAMBA-SQ. Two hundred specimens were tested by SAMBA-SQ and CAP/CTM. Ten discordant specimens were tie-broken by Abbott and genotyped to determine HIV-1 subtypes.

Of the 190 specimens withconcordant results, 92 hadVL resultsequal or above1000 copies/ml while 98 had VL resultsless than1000copies/ml. Among the specimens with discordant results, 2 had VL resultsequal or above1000 copies/ml on SAMBA while the corresponding CAP/CTM results were below1000 copies/ml. In addition, 8 had VL results below 1000 copies/ml on SAMBA while the corresponding CAP/CTM results were equal or above1000 copies/ml. Following tie-breaking of the 10 discordant specimenson the Abbott m2000 assay, the VL results were comparable to CAP/CTM hence they remained discordant compared to SAMBA. Therefore, against the reference CAP/CTM, SAMBA-SQ sensitivity was 92.0% (95% CI: 84.8, 96.5), specificity of 98.0% (95% CI: 93.0, 99.8), positive predictive value of 97.9% (95% CI: 92.5, 99.7), negative predictive value of 92.5% (95% CI: 85.7, 96.7) and an overall concordance of 90.0% (95% CI: 87.0, 92.3) with a Cohen Kappa value of 0.9 (95% CI: 0.84- 0.96) (Table 3).

Table 3: Comparison of SAMBA-SQ test performance with CAP/CTM(*n*=200) assessing sensitivity, specificity, PPV, NPV and agreement using Cohen Kappa analysis.

	CAP/CTM		
Viral load (cp/ml)	**≥ 1000**	**< 1000**	**Total**
SAMBA ≥	92	2	94
SAMBA <	8	98	106
Total	100	100	200
Sensitivity	92.0 (84.8-96.5)		
Specificity	98.0 (93.0-99.8)		
PPV	97.9 (92.5-99.7)		
NPV	92.5 (85.7-96.7)		
Concordance	90.0 (87.0-92,3)		
Cohen Kappa	0.9 (0.84-0.96)		

When the results were stratified to estimate performanceatfour VL categories, there was 55.6% discordance between SAMBA-SQ and CAP/CTM around the clinical cut off of 1000 copies/ml (Table 4).

Table 4: Comparison of SAMBA-SQ test at different viral load categories estimating the rate of concordance and discordance.

CAP/CTM

Viral load (cp/ml)	**Undetectable**	**<500**	**500-999**	**1000-1499***	**1500-2000**	**>2000**	**Total**
SAMBA ≥1000		1	1	4	4	84	94
SAMBA <1000	50	38	10	5	2	1	106
Total (%)	50	39	11	9	6	85	200
% Concordant	100	97.4	90.9	44.4	66.6	98.8	
% Discordant	0	2.6	9.1	55.6	33.4	1.2	

**VL range around the clinical cut off for treatment failure*.

4.1.3 HIV-1 Genotyping of discordant specimens

The rate of discrepancies between SAMBA and CAPCTM was 2% (11/535) with 90.9% (10/11) observed on SAMBA-SQ. Among the 11discordant specimens (1on EID and 9 on VL) between SAMBA and CAP/CTM10 were successfully genotyped. The remaining hadVL copies below 1000copies/ml (amplification sensitivity threshold of the in house genotyping assay). There were 60% (five AD and one AC) circulating recombinants forms while 40% were subtypes A(three A1 and one A) (Figure 10).

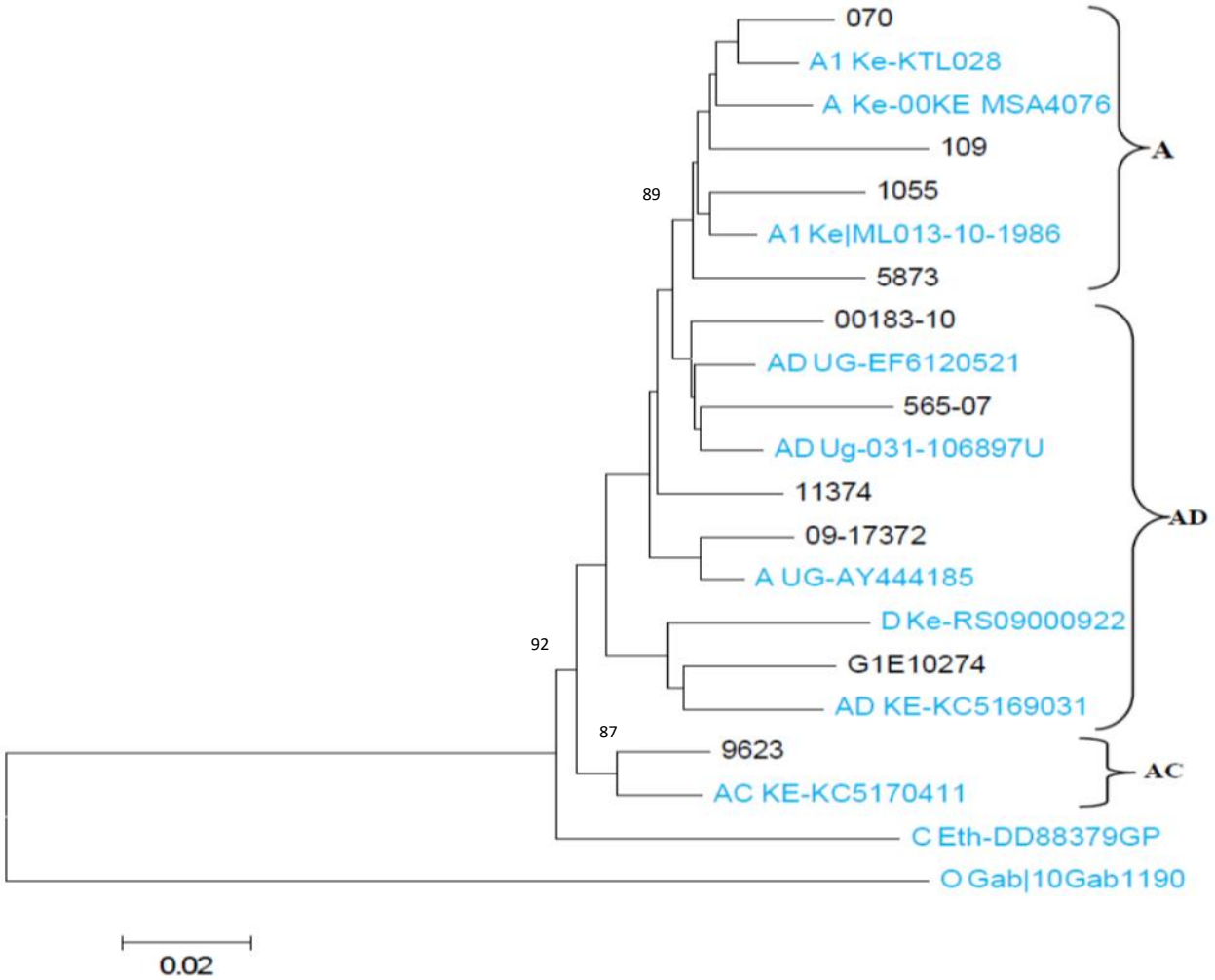


Figure 8: Phylogenetic analysis of the HIV-1*pol*gene sequences to determine the HIV subtypes in the ten successfully genotyped discordant specimens by neighbor-joining method using the GenBankdatabase subtype reference strains A, AD, AC, C and O in blue font (accession numbers). Boot- strap values ≥ 70% were considered significant.The characterized discordant specimen sequences in black fonts; pure subtypes(A and A1)and CRFs (AD and AC).

4.2 Discussion

Kenya, like other developing countries experience logistical and technical challenges limiting access and complicating HIV-1 molecular diagnostics. It will be vital to adopt POC technologies like SAMBA that will simplify, increase access and improve efficiency in diagnostics while maintaining the quality of patient care. Like many HIV-1 POC tests being designed for use in developed countries, SAMBA maynot be readily transferable in Kenya before evaluating itsaccuracy and reliability. This study therefore evaluated SAMBA for HIV-1 EID and VL monitoring in Western Kenya.

SAMBA-Q performance was comparable to the reference CAP/CTMassaywithhigh Cohen Kappa agreement of 0.99. This indicates that SAMBA-Q can be used to accurately diagnose HIV-1 in infants.In addition, the test can be performed within 2 hours;if utilized in peripheral health facilities it may encourage patients to remain on-site and appropriate clinical decision be made during the same visit. This is significantly beneficial in minimizing loss of patients due to follow up given the fact that patients frequently travel long distances to and from the health centers. This increases the potential of HIV-1 infected infants to access early diagnosis and be promptly initiated on antiretroviral therapy reducing aggressive progression of disease and mortality associated with untreated HIV-1 infection.

SAMBA-SQ performance was comparable to the reference CAP/CTM assay with high Cohen Kappa agreement of 0.9between the two platforms, this corroborates other findings(Ritchie *et al*., 2014).The data suggest that SAMBA-SQ with its clinical cut-off of 1000 copies/ml may prove useful in monitoring treatment response and for identifying patients who have developed virological failure and possible antiretroviral resistance. In addition, given that the test can be performed within 90 minutes, the patient can remain on-site and appropriate action can be taken during the same visitleading to improved outcomes for patients and overall public health.

Despite the comparable performance between the SAMBA and CAP/CTMassays, there were2% (11/535) discordant results indicating difference in accuracy. These findings are supported by reports from similar studies on SAMBA in Malawi and Uganda (Ritchie*et al*.,

2014).Thedifferencesin assays' accuracy have adverse clinical implications to patients' care. As it was observed with SAMBA-Q there was 0.3% (1/335) discordance. The ability of SAMBA-Q to detect HIV-1 in one specimen despite not being detected by CAP/CTM maylead to that infant being placed on ART promptly. Ifonly CAP/CTM was used for diagnosis, thisinfected infant would have had a false negative result hence not being initiated on ART. This may subsequently lead to death. Similarly, on SAMBA-SQ there was 1.0% (2/200) over estimation and 4% under estimation of VL. Like other quantitative assays, SAMBA-Q has a lower limit of estimation of 1000 copies/ml which is also the WHO clinical cut off for ART failure. Around this cut off, over 50% discordance was observed. This differing rate of reliability near the SAMBA-SQ cut off may have significant ramifications for individual patient ART monitoring. For example, under estimation of VL may lead to delay in detecting ART failure resulting in emergence of HIV-1 drug resistant strains. This may compromise patients' future treatment options. Equally VL overestimation may inflate ART failure rates in otherwise stable patients leading to possible patient and clinician anxiety. This may result to more frequent clinical visits, more laboratory testing and possibly unnecessary switches or intensification of ART. Several factors may be attributed to these differences in accuracy between the assays. Among these includepossible HIV-1 DNA/RNA degradation due to freeze-thaw leading to underestimation. Since the specimens were aliquoted to avoid freeze thawing this did not contribute to the discordant results in this studysince the specimens were detected by CAP/CTM and not SAMBA indicating presence of HIV nucleic acid. Other probable factorsinclude differences in specificity of the primers and assay target regionswhichmay be influenced by HIV-1 genetic diversity.

The HIV-1 subtypes and CRFs observed on the discordant specimens were A, AD and AC. These are among the HIV-1 subtypes reportedinKenya(Adungo*et al*., 2014; Khamadi*et al*., 2005; Oyaro *et al*., 2011; Yang *et al*., 2004). Although HIV-1 subtype A is dominant in Kenya, epidemiological studiesreport increased prevalence of A/C and A/D CRFs along the Kenya-Uganda border (Adungo, 2014; Kageha *et al*., 2012). The sustained circulation of recombinant strains AD and AC in Western Kenya as observed in this study and other previous studies support the hypothesis that conditions in this region supportHIV-1 genetic recombinationevents contributing to HIV-1 heterogeneity (Adungo*et al*., 2014; Khamadi*et a*l., 2005; Oyaro *et al*.,

2011; Yang *et al*., 2004).In view of the ongoing free interstate migration, there is a possibility of transfer of HIV-1 subtypes.This is demonstrated by the close homology of CRF AD with the Ugandan strains in the phylogenetic tree. These transfers may promote modification of viral diversity in these countries.Depending on the prevalence of HIV-1 subtypes,variation in accuracy between different HIV-1 PCR diagnostic assays have been documented (Jani*et al*., 2014; Korn*et al*., 2009; Luft*et al*., 2011; Taylor *et al*., 2009). While SAMBA has been optimized to detect HIV-1 subtypes A-G and a range of recombinant forms found in developingcountries (Lee *et al*., 2010) , the assay failed to detect some HIV-1 A, AD and AC strains.Similar studies have reported discordant results with subtype B and AD (Ritchie *et al*., 2014). This canbe attributed to the likely effect of high genetic variation in HIV-1 subtypes or extreme divergence within HIV-1 subtype and CRFs which SAMBA assay may have not been optimized to detect at the time of development.For example, the WHO panels used to optimize SAMBA assay did not contain CRF AC (Lee *et al*., 2010),this could explain the discordance for this specimen. In addition,differences in targeted regions also influencethe accuracy of HIV-1 diagnostic assays (Taylor *et al*., 2009).In this case SAMBA primers target the LTR region while CAP/CTM uses the multiplex approach targeting both the LTR and *gag* regions to improve its sensitivity(Huang *et al*., 2008). Having compared these two technologies, studies have reported difference in accuracy. These variations were attributed to the existenceof natural polymorphisms occurring in primer/probe sequences that have the potential to reduce or abolish hybridization during PCR.(Korn*et al*., 2009; Luft*et al*., 2011; Swanson *et al*., 2005; Wirden*et al*., 2009).Consequently genetically divergent HIV-1 variants may not be optimally detected by HIV-1 PCR diagnostic assays since more often not all subtypes and target sequence information is known at the time of testing.This was observed with SAMBA and the overall implication in larger Kenyan population may need to be established. Therefore appreciation of the limitations of PCR assays' inability to detect all existing or emerging strains is imperative when making decisions for clinical management of HIV infected patients.

In summary, this evaluation showedthe performance of SAMBA to be comparable to CAP/CTM.

4.2.1 Study limitations

i. Since this was a cross sectional study, patients demographics for example, when infected, $CD4_+$ T lymphocyte counts, treatment history could not be obtained.

ii. Despite random sampling which minimizes bias, sample size calculation was based on the former Nyanza region HIV prevalence, rather than each of the counties' with possible difference in prevalence.

iii. Since HIV-1 heterogeneity is diverse and with the possibility of regional subtype transfer, samples obtained from Western Kenya may not be representative of other regions of the country especially those bordering other countries.

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

i. SAMBA-Q showed comparableperformance characteristics (sensitivity and specificity) and concordance to CAP/CTM.

ii. SAMBA-SQ showed comparable performance characteristics (sensitivity and specificity) and concordancetoCAP/CTM.

iii. HIV-1 subtypes in Western Kenya did not significantly influence the accuracy of SAMBA-Q and SAMBA-SQ.

5.2 Recommendations

i. SAMBA-Qshould be considered for adoption to contribute towards decentralization of HIV-1early infant diagnosis in Western Kenya.

ii. SAMBA-SQshould be considered for adoption to contribute towards decentralization of HIV-1 VL monitoring in Western Kenya.

iii. Due to the discordance observedbetween CAP/CTM and SAMBA, there is need for continuous evaluation in larger populations and optimization of SAMBA assays' targetto enhance its accuracy in diagnosis of the highly variable and continuously mutating HIV.

5.3 Future Research

Implementation research studies that need to be conducted to enable adoption of SAMBA as a POC in Kenya include:

i. Country wide study to determine the role of circulating HIV-1 subtypes on SAMBA's accuracy

ii. A feasibility study to determine integration of SAMBA in local health facilities. Among the factors that will be assessed will include it's accuracy in other parts of the country, ease of use by non-laboratory healthcare workers and possible logistical challenges when using it.

iii. Study to compare the proportion of patients retained in care and treatment between those tested using SAMBA and referral laboratory testing systems.

iv. A cost-effectiveness analysis of using the POC SAMBA system at health facilities compared to using referral EID and VL systems at centralized laboratories.

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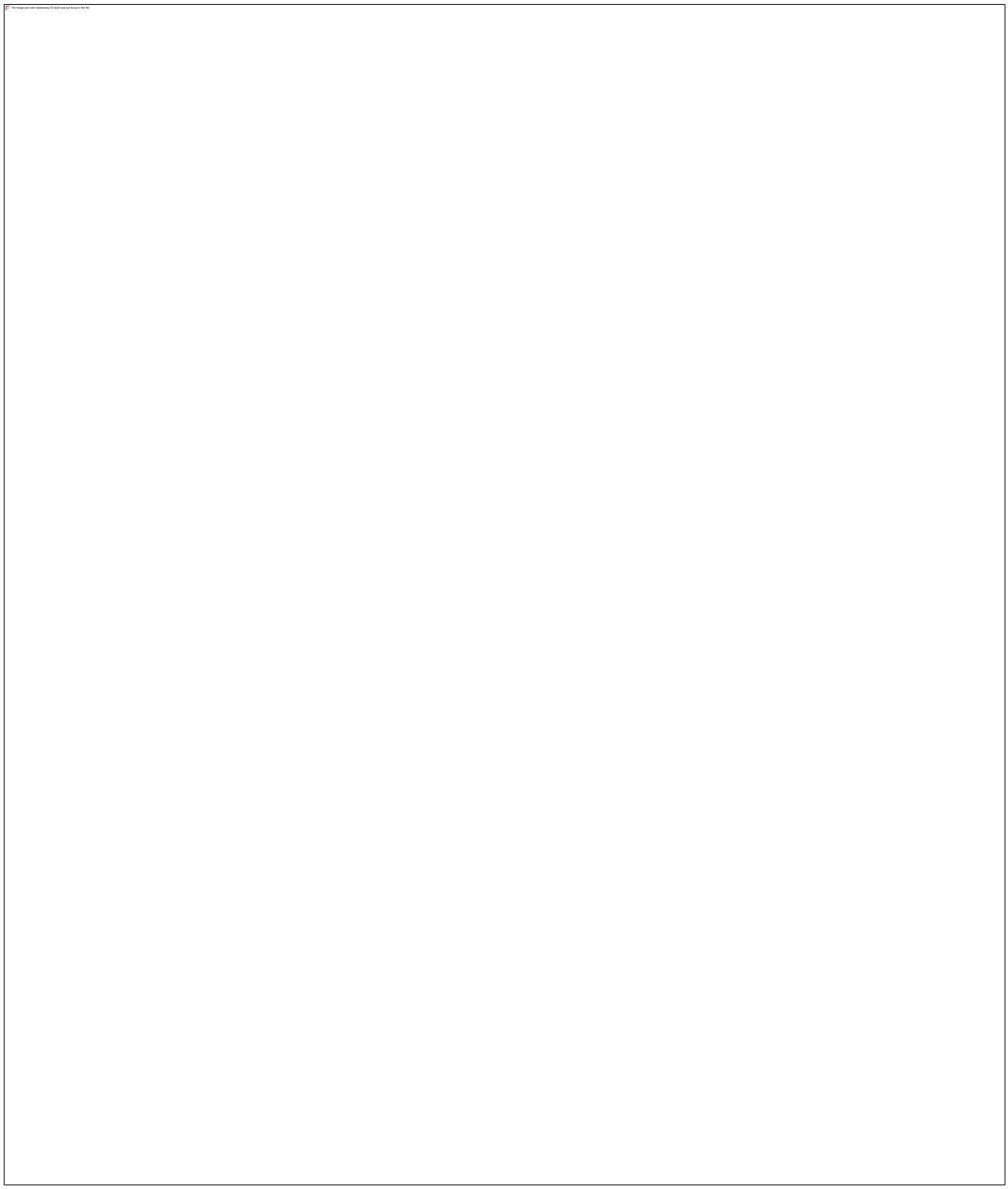
APPENDICES

Appendix 1: Study Ethical Clearance



Appendix 2: Parents/ guardians consenting form

Parental/guardianConsent to participate in SAMBA Qualitative (EID) Study

A Randomized Controlled Trial of New Point-of-Care Tests for Early Infant Diagnosis and Viral Load Monitoring of HIV-1 Infection in Resource-poor Settings in Africa

Parental Consent to Take Part in SAMBA Qualitative (EID)

For:

- **Infants (18 months or below)**

INTRODUCTION:

Permission is being sought for your dependent/child to take part in medical research. Research is also called a study. A medical study can look at what causes a disease. It can also look at ways to prevent or control a disease. The research is being done by Kenya Medical Research Institute/U.S Centers for Disease Control and Prevention (KEMRI/CDC). Before you can decide if you want your dependent/child to take part, we would like to tell you more about the study. We will tell you about:

- What your dependent/child will need to do to take part
- Risks to your dependent/child if s/he takes part
- How this research might help her/him
- How we will keep her/his information private
- Your dependent/child's rights to leave the study
- What you can do if you do not want your dependent/child to take part
- Who you can talk to if you have problems or questions

PURPOSE OF THE STUDY:

This study is being conducted to compare and identify the relative strengths and weaknesses of the new SAMBA Early Infant Diagnosis (EID) testing device that can give rapid same-day results at the point of care as compared to existing laboratory-based EID tests using venous blood and dried blood spots (DBS) in de-identified HIV infected patient specimens. The specific

purpose of this phase of the study is to determine the feasibility of using the new SAMBA point of care EID system among field based staff like laboratory technicians and technologists compared to ideal laboratory conditions for EID.

WHO WILL TAKE PART:

To be in this study, your dependent/child must:

- Be residents of Siaya County.
- Be aged 18 months or below
- Be born to HIV infected mother and/or previously identified as PCR positive

TAKING PART IS VOLUNTARY:

It is important to know the following:

- No one can make your dependent/child take part if you do not want to.
- Learn all the facts about the study before you make a choice.

We would like you to ask us any questions you have. We would also like you to tell us when you do not understand something. You may take whatever time you need to make your choice.

If you allow your dependent/child to take part, you will be asked to sign this consent form. You will be given a copy to keep.

It is important for you to know that even if you allow your dependent/child to take part in the study:

- S/he may leave at any time.
- You may choose not to answer certain questions.
- Your child/dependent will still receive medical care and other benefits from other studies with KEMRI/CDC even if s/he does not take part in this one.
- S/he will still get her/his regular tests and the usual care if you choose not to allow him/her to be in the study.

HOW LONG YOUR DEPENDENT/CHILD WILL BE IN THE STUDY:

If you allow your dependent/child to take part in the study, she/he will have one study visit today. This is called the screening/enrolment visit. It will take about one-and-a-half hours to complete this visit. At this time, you will be told if your dependent/child is suitable for the study. If your dependent/child is suitable and you agree that she takes part, we will offer your dependent/child EID test and we will ask you to come back for the results.

WHAT WILL HAPPEN IN THE STUDY:

If you permit that your dependent/child should take part in the study, we will draw about 1 ml of blood for the EID procedure. Your dependent/child's blood will tested at the facility by the site staff using the SAMBA EID device, the leftover blood will be taken to the KEMRI/CDC HIV research laboratory in Kisumu for processing using the regular devices. The results from the KEMRI/CDC HIV research laboratory will be availed to you on the next clinic visit, and will be used for your dependent/child's management. Your dependent/child's blood will not be sold or used for transfusions. It will only be used for research purposes in this study. A staff member will explain how this needs to be done. The EID test will show whether the HIV virus is in your dependent/child's blood or not.

IF YOU CHOOSE FOR YOUR DEPENDENT/CHILD NOT TO TAKE PART:

If you choose for your dependent/child not to take part in this study, there will be no harm /risk at all and your participation in other research studies will not be interfered with. We would like to ask you some questions about your decision. A member of study staff will enter your answers into the computer. It will only take a few minutes to answer these questions. Remember, you can choose not to answer these questions.

RISKS /DISCOMFORT:

- During the blood draw procedure your dependent/childmay feel pain on her/his finger/heel. Your dependent/childmay bruise and thereis a small chance of an infection where the blood is taken from. Clinical staff will follow proper procedures to lessen this risk.
- People in your community, including your family, may learn that your dependent/childis taking part in this study. Some of them may not be pleased that your dependent/childis doing so.

BENEFITS:

- You and your community may learn more about HIV because you have taken part in this study
- In the long term, the availability of point-of-care Early Infant HIV diagnosis tests will benefit all HIV exposed children.

PRIVACY/CONFIDENTIALITY:

All information for your dependent/child will be kept private by the study staff. Findings from this study will use information from everyone who took part. It will not focus just on your dependent/child's medical test results. Overall findings from this study will be shared with the community. Nothing about your dependent/child specifically will be included in these findings. Your dependent/child will be given a special study number. This number will be used on all her/his study records. Your dependent/child's name will not be on any of these records. Your dependent/child's name and personal information will only be used to reach her/him. It will not be included in any reports.

YOUR RIGHTS AND YOUR DEPENDENT/CHILD'S RIGHTS TO REFUSE TO TAKE PART IN THE RESEARCH OR LEAVE THE RESEARCH:

You may choose not to allow your dependent/child to take part in this study, this will not affect the medical care that s/he has been receiving before the study and it will not affect your dependent/child's participation in other KEMRI/CDC research. You may choose to answer some questions and not to answer others. Your dependent/child may stop taking part at any time.

REASONS WHY YOUR DEPENDENT/CHILD MAY BE WITHDRAWN FROM THE RESEARCH WITHOUT YOUR CONSENT:

- KEMRI/CDC can stop the study at any time.
- Your dependent/child can also be removed from the study. This may be done if there is concern that the research may harm her/him.

ALTERNATIVES IF YOUR DEPENDENT/CHILD DOES NOT TO TAKE PART:

If you choose not to allow your dependent/child to take part in this study, s/he is free to continue with his/her participation in other KEMRI/CDC research studies and also take part in other upcoming research. We shall provide you with information on other health care sites that offer HIV counselling, care and treatment.

COST TO YOU OR YOUR DEPENDENT/CHILD:

Thereis nocosttoyour dependent/childfor being inthe study. At the end of every study visit, we will offer your dependent/child a bar of soap.

PROBLEMS OR QUESTIONS:

Ifyou havequestions about thisresearch orfeel that your dependent/child has been harmed by the research, please contact Clement Zeh or Frank Angira at the KEMRI/CDC Research Field station at Kisian, Kisumu. (P.O. Box 1578, Kisumu) at telephone 057-2022902/59/83 or cell phone +254 721653270.

Ifyou have questions about you or your dependent/child'srights,you can contact Dr. John Vulule, KEMRI director, at the KEMRI/CDC Research Field Station, (P.O. Box 1578, Kisumu) at telephone 057-2022924/22940 or through 057-2022902/59/83. You may also contact the Secretary of the KEMRI National Ethical Review Committee, Nairobi at telephone 020-2722541.

The above numbers are not for emergencies. If you are having an emergency, please go to the nearest clinic.

Do you have any questions?

STATEMENT OF CONSENT:

I have readand/or had thisform readtome. I understand the purpose of the research. All the procedures have been explained to me and my questions have been answered.

☐*I give permission for my dependent/child [insert name] to be in the research*

☐*I **do not** give permission for my dependent/child [insert name] to be in the research.*

I understand that my dependent/child's test results will be accessed.

☐*I give permission for my dependent/child's test results to be accessed.*

☐*I **do not** give permission for my dependent/child's test results to be accessed.*

Parent/Guardian's Name: (Print)		Parent/Guardian's signature:		Date:	
Witness' Name: (Print)		Witness' Signature:		Date:	

I have explained the purpose of this research to the volunteer. To the best my knowledge, he/she understands the purpose, procedures, risks and benefits of this research.

Investigator's or designee's Name: (Print)		Investigator's or designee's signature:		Date:	

Appendix 3: Adult consenting form

Consent to participate in SAMBA Viral Load Evaluation Study

A Randomized Controlled Trial of New Point-of-Care Tests for Early Infant Diagnosis and Viral Load Monitoring of HIV-1 Infection in Resource-poor Settings in Africa

Consent to Take Part in SAMBA Viral Load Evaluation Study

For:

- **Adults (≥ 18years)**
- **Mature Minors (< 18years and pregnant, married, or a parent)**

INTRODUCTION:

You are being asked to take part in medical research. Research is also called a study. A medical study can look at what causes a disease. It can also look at ways to prevent or control a disease. The research is being done by KEMRI/CDC. It will look at men and women with the Human Immunodeficiency Virus (HIV) infection who are seeking for Viral Load test as part of care and treatment requirements. Before you can decide if you want to take part, we would like to tell you more about the study. We will tell you about:

- What you will need to do to take part
- Risks to you if you take part
- How this research might help you
- How we will keep your information private
- Your rights to leave the study
- What you can do if you do not want to take part
- Who you can talk to if you have problems or questions

PURPOSE OF THE STUDY:

This study is being conducted to compare and identify the relative strengths and weaknesses of the new SAMBA Viral load (VL) testing device that can give rapid same-day results at the point of care as compared to existing laboratory-based viral load tests using venous blood in de-

identified HIV infected patient specimens. The specific purpose of this phase of the study is to determine the feasibility of using the new SAMBA point of care VL system among field based staff like laboratory technicians and technologists compared to ideal laboratory conditions in measurement of VL.

WHO WILL TAKE PART:

To be in this study, you must:

- Be HIV infected and is receiving care and treatment at an HIV care and treatment facility
- Be willing to provide written informed consent or assent

TAKING PART IS YOUR CHOICE:

It is important to know the following:

- No one can make you take part if you do not want to
- Learn all the facts about the study before you make a choice

We would like you to ask us any questions you have. We would also like you to tell us when you do not understand something. You may take whatever time you need to make your choice. It is important that you fully understand what the study will ask of you.

It is important for you to know that even if you choose to take part in the study:

- You may leave at any time
- You may choose not to answer certain questions

If you choose not to take part in the study, you will still receive medical care and other benefits from other studies with KEMRI/CDC and you will still get your regular tests and the usual care.

HOW LONG YOU WILL BE IN THE STUDY:

If you choose to take part in the study, you will have one study visit today. This is called the screening/enrolment visit. It will take about 45 minutes to complete this visit. At this time, you will be told if you are suitable for the study. If you are suitable and agree to take part, we will ask you to participate only once. If you are not suitable for the study or decline study participation, you will not be asked to take part in the study.

WHAT WILL HAPPEN IN THE STUDY

If you decide to take part in the study you will be asked to give about 3-4ml of blood (about a teaspoonful) for a Viral load test using the SAMBA assay at the clinic and a leftover of the blood taken to the KEMRI/CDC HIV research laboratory in Kisumu for processing using the regular centralized Viral load assays. The results from the HIV Research laboratory will be availed to you on your next clinic visit, results from the SAMBA point of care will not be released for clinical management. Your blood will not be sold or used for transfusions. It will only be used for research purposes in this study. A staff member will explain how this needs to be done. The VL result will show the number of virus in your body and how much the disease has advanced in you.

IF YOU CHOOSE NOT TO TAKE PART:

If you choose not to take part in this study there will be no harm /risk at all and your participation in other research studies will not be interfered with.

RISKS /DISCOMFORT:

- You may feel uncomfortable, particular attention will be made to avoid this discomfort
- During the blood draw procedure you may feel pain. You may bruise and there is a small chance of an infection where the blood is taken from. Clinical staff will follow proper procedures to lessen this risk.
- People in your community, including your family, may learn that you are taking part in this study, some of them may not be pleased that you are doing so. All study staff will maintain high level of confidentiality.

BENEFITS:

- You and your community may learn more about HIV because you have taken part in this study
- In the long term, the availability of point-of-care test will benefit all HIV patients in treatment programs

PRIVACY/CONFIDENTIALITY:

All information obtained will be kept private by the study staff. No one else will be told your answers to questions or results of medical tests. Findings from this study will use information from everyone who took part. It will not focus just on your answers and medical test results. Overall findings from this study will be shared with the community. Nothing about you specifically will be included in these findings.

You will be given a special study number. This number will be used on all your study records. Your name will not be on any of these records. Your name and personal information will only be used to reach you. It will not be included in any reports.

YOUR RIGHTS TO REFUSE TO TAKE PART IN THE RESEARCH OR LEAVE THE RESEARCH:

You may choose to take part in this study or you can choose not to take part in it. If you choose not to take part, your medical care that you have been receiving before the study will not be affected. It will not affect your participation in other KEMRI/CDC research. You may choose to answer some questions and not to answer others. You may stop taking part at any time.

REASONS WHY YOU MAY BE WITHDRAWN FROM THE RESEARCH WITHOUT YOUR CONSENT:

- KEMRI/CDC can stop the study at any time
- You can also be removed from the study. This may be done if there is concern that the research may harm you

ALTERNATIVES IF YOU CHOOOSE NOT TO TAKE PART:

If you choose not to take part in this study, you are free to continue with your participation in other KEMRI/CDC research studies and also take part in other upcoming research. We shall provide you with information on other health care sites which offer HIV counselling, care and treatment.

COST TO YOU:

There is no cost to you for being in the study. At the end of this study visit, we will offer you a bar of soap.

PROBLEMS OR QUESTIONS:

If you have questions about this research or feel you have been harmed by the research, please contactClement Zeh or Frank Angira at the KEMRI/CDC Research Field station at Kisian, Kisumu. (P.O. Box 1578, Kisumu) at telephone 057-2022902/59/83 or cell phone +254 721653270.

If you have questions about your rights, you can contact Dr. John Vulule, KEMRI director, at the KEMRI/CDC Research Field Station, (P.O. Box 1578, Kisumu) at telephone 057-2022924/22940 or through 057-2022902/59/83. You may also contact the Secretary of the KEMRI National Ethical Review Committee, Nairobi at telephone 020-2722541.

The above numbers are not for emergencies. If you are having an emergency, please go to the nearest clinic.

Do you have any questions?

STATEMENT OF CONSENT:

I have read and/or had this form read to me. I understand the purpose of the research. All the procedures have been explained to me and my questions have been answered.

☐ *I agree to participate in the research.*

☐ *I **do not** agree to participate in the research.*

I understand that my test results will be accessed.

☐ *I agree for my test results to be accessed.*

☐ *I **do not** agree for my test results to be accessed.*

Participant's Name: (Print)		Participant's signature:		Date:	
Witness' Name: (Print)		Witness' Signature:		Date:	

I have explained the purpose of this research to the volunteer. To the best my knowledge, he/she understands the purpose, procedures, risks and benefits of this research.

Investigator's or designee's Name: (Print)		Investigator's or designee's signature:		Date:	

Appendix 4: EID patient Screening Form

EID Patient Screening Form

A Randomized Controlled Trial of New Point-of-Care Tests for Early Infant Diagnosis and Viral Load Monitoring of HIV-1 Infection in Resource-poor Settings in Africa

Study ID number: ||_|__|__|__| _____Hospital ID number - Infant: _____

_____Hospital ID number – Parent/Guardian: _____

Date of screening visit: |__|__| / |__|__| / |__|__|__|__|

Age: |__|__| **months** Sex: ☐ male ☐ female

Inclusion criteria (all must be YES):	**YES**	**NO**
• Born to HIV-1 positive mother or Infant 18 months old or below, previously tested as HIV-1 positive by DBS-PCR ……	☐	☐
• Infant 18 months old or below ……	☐	☐
• Parent/Guardian willing to provide consent ……	☐	☐
Exclusion criteria (if the answer is NO to any one of the following criteria)		
• Born to an HIV-1 positive mother……	☐	☐

Appendix 5: VL Patient Screening Form

VL Patient Screening Form

A Randomized Controlled Trial of New Point-of-Care Tests for Early Infant Diagnosis and Viral Load Monitoring of HIV-1 Infection in Resource-poor Settings in Africa

Study ID number: ||_|__|__|__| _____Hospital ID number:

_____

Date of screening visit: |__|__| / |__|__| / |__|__|__|__|

Age: |__|__| **Months** |__|__| **Years** Sex: ■ male ■ female

Inclusion criteria (all must be YES):	**YES**	**NO**	**N/A**
• HIV-1 infected individuals requiring viral load test for clinical management	■	■	
• Participant or Parent/Guardian for children/minors willing to provide consent	■	■	
• Participant (children/minor) willing to provide assent	■	■	☐
Exclusion criteria (if the answer is NO to any one of the following criteria)			
• HIV-1 infected individual	■	■	

Appendix 6: Testing procedures for HIV EID and VL using Roche CAP/CTM

HIV-1 RNA VL and EID PCR using COBAS Ampliprep/COBAS TaqMan

1.1 Aliquot 1100ul of plasma for VL and 100ul of whole blood for EID in the appropriately labeled S-tubes. If the sample volume
1.2 Vortex 1 vial of CTM (—) C thoroughly for 20 seconds and transfer 1100µl to an input S-tube marked 1; NC, and recap the s-tube.
1.3 Vortex 1 vial of HIV-1 L (+) C thoroughly for 20 seconds and transfer 1100µl to an input S-tube marked 2; PC, and recap the s-tube
1.4 Attach 21 barcode label clips to each SK24 rack position 4-24 for VL (3-24 for EID) where a Sample input S-tube is to be placed. Check the seating of the clips on the rack.
1.5 Attach a CTM (—) C (negative control) barcode label clip blue in color on a SK24 rack position 1.
1.6 Attach a HIV-1 L (+) C (low positive control) barcode label clip yellow in color on a SK24 rack position 2.
1.7 For VL Attach a HIV-1 H (+) C (High positive control) barcode label clip yellow in color on a SK24 rack position 3.
1.8 Requisition the work order on the Amplilink data station.
- 1.8.1 click on orders → sample rack → new → (assign rack ,batch ID as plate number and comments as kit lot number)
  - **1.8.1.1** select appropriate test file – HI2CAP96 for VL and HI2QUAL96 for EID.
  - **1.8.1.2** Click on NC, then LPC, HPC enter patient IDs, save and print.
  - **1.8.1.3** Printed sample rack order report is to be used as a worksheet.

1.9 Make sure that the S-tube caps are finger tight loosened.
1.10 Load the SK 24 rack(s) onto rack positions **F**, **G** or **H** the Ampliprep machine.
1.11 Start the run by pressing **START** on the System folder
1.12 COBAS TaqMan will start the run automatically and therefore no intervention necessary.
1.13 At the completion of the COBAS TaqMan analyzer, check for flags or error messages on the result report.
1.14 Review and accept results, then print.
1.15 Remove instrument waste. If there is no any sample to run,
- 1.15.1 If Reagent cassettes have remaining reagent as noted on system Status screen, remove cassettes and place in refrigerator in box labeled with tests remaining, initials, and date used.
- 1.15.2 Remove and discard waste from COBAS® AmpliPrep instrument.
- 1.15.3 Waste bottle should be emptied down laboratory sink drain, followed by copious amounts of water.
- 1.15.4 Used SPUs, reagent cassettes, and S-input tubes should be discarded into 5- gallon containers lined with a biohazard bag.

1.16 If docking system is not connected, transfer the processed samples manually to the TaqMan for the detection and analysis.
- 1.16.1 **Note:** Processed samples containing master mix solution have limited stability and should be immediately transferred for analysis within 120 minutes.

1.16.2 **Note:** After completion of sample preparation, all processed samples and controls should not be exposed to light.

1.16.3 **Note:** do not freeze or store processed samples at 2-8°C

1.17 The K-carrier rack is locked and can only be removed when the processing is complete and the light turns orange.

1.18 Open the COBAS Ampliprep instrument load panel. Do not stare into a laser transmitter.

1.19 Remove the sample rack containing the processed samples. Do not separate k-tubes from the barcode clips; keep them in the sample rack.

1.20 Close the COBAS Ampliprep instrument load panel.

1.21 Open the COBAS TaqMan analyzer load panel, and slide the rack containing the processed samples onto one of the four sample rack positions.

1.22 Close the load panel.

1.22.1.1 If the rack is properly loaded and matching work orders are found, k-tubes are moved to a k-carrier and the k-carrier is loaded into an available thermal cycler segment.

1.22.1.2 If no thermal cycler segments are available the k-carrier is moved to a park position on the right side of the analyzer.

1.23 If samples are properly loaded and matching work orders are found, the run starts automatically.

1.24 At the completion of the COBAS TaqMan analyzer, check for flags or error messages in the result report.

1.25 Review and accept results, then print.

1.26 Results Interpretation.

Titer Result	**Interpretation for VL**
Target Not Detected	Ct value for HIV-1 above the limit for the assay or no Ct value for HIV-1 obtained. Report results as "HIV-1 RNA not detected
<2.00E+01 cp/mL	Calculated cp/mL are below the Limit of Detection of the assay. Reportresults as "HIV-1 RNA detected, less than 20 HIV-1 RNA cp/mL ".
' 2.00E+01 cp/mL and 1.00E+07 cp/mL	Calculated results greater than or equal to 20 cp/mL and less than or equal to 1.00E+07 cp/mL are within the Linear Range of the assay..
> 1.00E+07 cp/mL	Calculated cp/mL are above the range of the assay. Report results as"greater than 1.00E+07 HIV-1 RNA cp/mL ". If quantitative results are desired, the original specimen should be diluted 1:100 with HIV-1-negative human EDTA-plasma and the test repeated. Multiply the reported result by the dilution factor.

Result	**Interpretation for EID**
Target Not Detected	"HIV-1 RNA/DNA not detected
Target Detected	"HIV-1 RNA/DNA detected

Appendix 7: Testing procedures for HIV EID and VL using Abbott m2000

Abbott m2000 Real-Time HIV-1 VL and EID PCR

1.1. Transfer 600ul of plasma for VL or 100ul whole blood for EID into 5 mL reaction vessel
1.2. Thaw assay controls and IC at 15-30°C.
1.3. Once thawed, assay controls and IC can be stored at 2-8°C for up to 24 hours before use.
1.4. Vortex each assay control three times for 2-3 seconds before use.
1.5. Ensure that the contents of each vial are at the bottom after vortexing by tapping the vials on the bench to bring liquid to the bottom of the vial.
1.6. Ensure bubbles or foam is not generated; if present, remove with a sterile pipette tip, using a new tip for each vial.
1.7. Thaw amplification reagents at 15-30°C or at 2-8°C and store at 2-8°C until required for the amplification master mix procedure.
1.8. Once thawed, the amplification reagents can be stored at 2-8°C for up to 24 hours if not used immediately.
1.9. Open the *m*Sample Preparation System reagent pack(s).Incase of crystals allow to dissolve by gently inverting the bottle continuously.
1.10. Vortex each IC vial three times for 2-3 seconds before use. Use a calibrated pipette to add 500 µL of IC to each bottle of *m*Lysis Buffer. Mix by gently inverting the container 5 to 10 times to minimize foaming.
1.11. Gently invert all the reagent bottles except *m*Microparticle 5 to 10 times to ensure a homogenous solution and pour the contents into the appropriate 200ml reagent vessels. Ensure bubbles or foams are not generated for all the reagent vessels; if present, remove with a sterile pipette tip, using a new tip for each reagent vessel.
1.12. Place the negative control, low positive, high positive control and calibrators if applicable in each run and the patient specimens into the Abbott *m2000sp* sample rack.
1.13. Load specimens and controls into the 13 mm sample racks in consecutive positions.
1.14. Insert specimen and control tubes into sample racks carefully to avoid splashing. If used, bar codes on tube labels must face right for scanning.
1.15. Ensure that each tube is placed securely in the sample rack so that the bottom of the tube reaches the inside bottom of the rack.
1.16. Load filled sample racks onto the Abbott *m2000sp* in consecutive sample rack positions, with the first rack farthest to the right on the worktable, and any additional rack progressively to the left of the first rack
1.17. Place the 5 mL Reaction Vessels into the Abbott *m2000sp* 1 mL subsystem carrier.
1.18. Load the Abbott 96 Deep-Well Plate on the Abbott *m2000sp* worktable as described in the Abbott *m2000sp* Operations Manual, Operating Instructions section.
1.19. From the Protocol screen, select the appropriate application file.
1.20. Initiate the sample extraction protocol as described in the Abbot*t m2000sp* Operations Manual, Operating Instruction section.
1.21. For VL enter calibrator (needed if a calibration curve has not been stored on the m2000rt, refer to 7.2.Assay Calibration) and control specific values in the sample extraction: worktable setup, calibrator and control fields. Lot specific values are specified in each Abbott RealTime HIV-1 Calibrator and control kit card.

1.22. Initiate the sample extraction protocol as described in the Abbott *m*2000*sp* Operations Manual, Operating Instruction section

1.23. At the end of the Sample Extraction Protocol, close the process and proceed with the Master Mix Addition protocol.

NOTE: The Abbott *m*2000*sp* Master Mix Addition protocol must be initiated within one hour after completion of Sample Preparation..

1.24. Load the amplification reagents and the Master Mix Tube on the Abbott *m*2000*sp* worktable after sample preparation is completed.

1.24.1. Each Amplification Reagent Pack supports up to 24 reactions.

1.24.2. Prior to opening the amplification reagents, ensure that the contents are at the bottom of the vials by tapping the vials in an upright position on the bench.

1.24.3. Remove and discard the amplification vial caps.

1.24.4. Place the 96 well Optical reaction plate.

1.25. From the Protocol screen, select the appropriate application file corresponding to the sample volume being tested.

1.26. Initiate the Abbott *m*2000*sp* Master Mix Addition protocol. Follow the instructions as described in the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.

NOTE: The Abbott *m*2000*rt* protocol must be started within 50 minutes of the initiation of the Master Mix Addition protocol.

1.27. Switch on and initialize the Abbott m2000rt instrument in the Amplification area immediately after the master mix addition process has been started.

NOTE: The Abbott *m*2000*rt* requires 15 minutes to warm-up.

1.28. At the end, immediately close the process after the Abbott m2000sp instrument has completed addition of samples and master mix according to the Abbott *m*2000*sp* Operations Manual, Operating Instructions section.

NOTE: Do not touch the surface or the bottom of the plate

1.29. Seal the Abbott 96-well optical reaction plate using the Abbott Optical Adhesive Cover.

1.30. Use applicator to sufficiently press the seal on the reaction plate then remove the Abbott 96-Well Optical Reaction Plate and place it onto the Abbott Splash Free Support Base

1.31. To send the plate information *m*2000rt instrument, view Results of the corresponding PCR plate then Export to Network Drive or burn on CD before proceeding to the Amplification Area.

1.32. At the Abbott *m*2000*rt* instrument protocol screen, select the appropriate application file, before initiating the Abbott RealTi*m*e HIV-1 protocol, as described in the Abbott *m*2000*rt* Operations Manual, Operating Instructions section. Alternatively, if the plate has been exported from *m*2000sp instrument, From the Orders, Import the appropriate plate from the Network Drive or insert the burned CD and import the data. Set Up Run. You will be prompted to place the Abbott 96-Well Optical Reaction Plate in the Abbott *m*2000*rt* instrument before setting the run to start.

NOTE: If creating the *m*2000*rt* test order manually, enter sample IDs in the corresponding PCR tray locations according to the "wells for

selected plate" grid found on the detail screen of the "PCR plates results" on the *m2000sp*.

1.33. At the completion of the run, assay results are reported on the Abbott *m2000rt*.

1.34. Remove the 96 deep-well plate from the worktable and dispose according to the Abbott *m2000rt* Operations Manual.

1.35. Place the Abbott 6 deep well plate in a sealable plastic bag and dispose according *to the* Abbott *m2000rt* Operations Manual, Operating Instructions section.

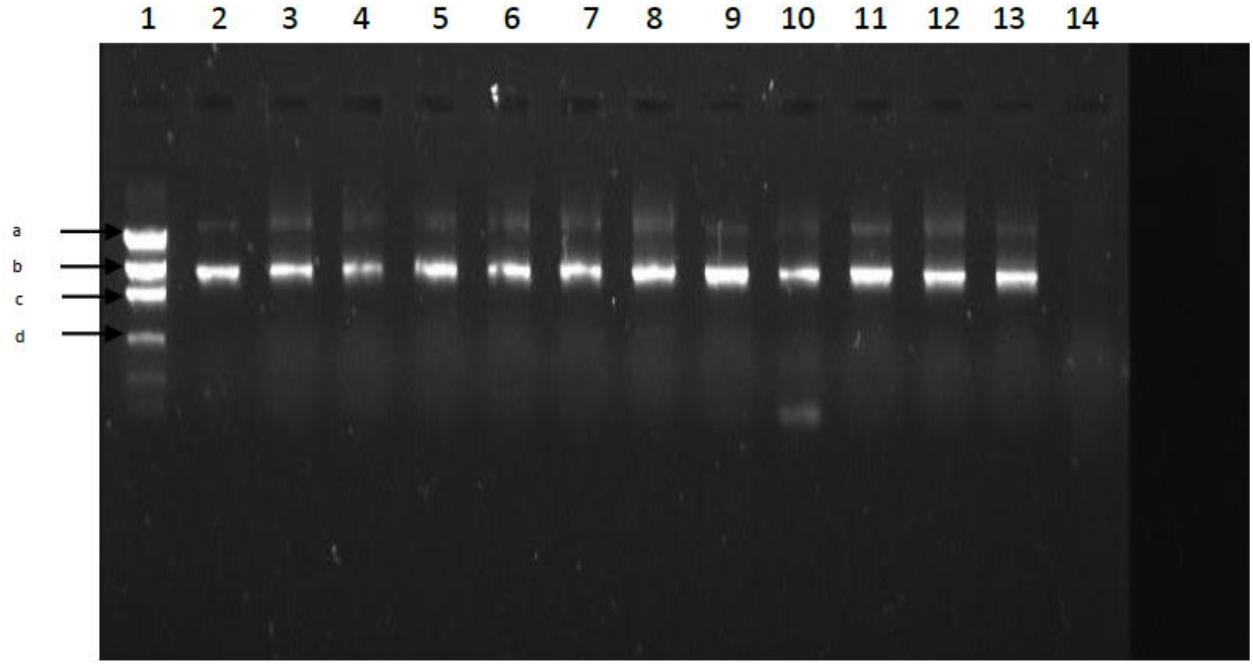
1.36. Clean the splash free support base before next use, according to the Operations Manual.

1.37. Results Interpretation.

VL application file result	Interpretation
Target Not Detected	<1.60 Log (Copies/mL)
Target Detected	1.60 to 7.00 Log (Copies/mL)

VL application file result	Interpretation
Target Not Detected	"HIV-1 RNA/DNA not detected
Target Detected	"HIV-1 RNA/DNA detected

Appendix 8: Gel electrophoresis images



Gel confirmation of PCR amplification using 1% agarose gel electrophoresis and 6ul mass ladder. Position 1 is the mass ladder (bands; a) 2.0kb, b) 1.2kb, c) 0.8kb and d) 0.4kb. Bands 2 to 12 (specimen IDs;070, 9623, 11374, 1055, 109, 565-07, 09-17372, G1E10274, 5873, and 00183-10)represent the specimen PCR amplicons. Band 13 represents the known kit Positive controland band 14 known kit Negative control.