EFFECTS OF DEPOMEDROXYPROGESTERONE ACETATE (DMPA) ON SYSTEMIC AND LOWER GENITAL TRACT HIV 1 VIRAL LOADS IN HIV POSITIVE ANTI RETROVIRAL THERAPY NAÏVE WOMEN ATTENDING A COMPREHENSIVE CARE CENTRE IN KISUMU KENYA: A CASE CONTROL STUDY.

A dissertation submitted to the University of Nairobi in part fulfilment of the degree of Master of Medicine in Human Pathology

PRINCIPAL INVESTIGATOR:

DR. WAITHERA NJENGA GITHENDU

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DECLARATION

This dissertation represents an original study, and has not been presented to any other institution for review and approval.

DR. WAITHERA NJENGA GITHENDU
MB; ChB (UoN)
Resident, Department of Human Pathology,
University of Nairobi
PO Box 19676-00202,
Nairobi, Kenya
Email:alisonegithendu@gmail.com

SIGNATURE _____ DATE _____

APPROVAL

We confirm that the work reported in this thesis was carried out by the candidate under our supervision. It is submitted for award of Masters Degree in Human Pathology from University of Nairobi with our approval as university supervisors.

SUPERVISORS:

1. PROF. WALTER JAOKO MB;ChB, MTropMed, PhD

Professor,

Department of Medical Microbiology,

School of Medicine,

College of Health Sciences,

University of Nairobi

PO Box 19676-00202

Nairobi, Kenya

E mail: wjaoko@kaviuon.org

Signature: _____ Date: _____

2. PROF. CHRISTINE KIGONDU BSc, PhD

Associate Professor,

Thematic unit of Clinical Chemistry.

Department of Human Pathology

School of Medicine,

College of Health Sciences,

University of Nairobi

P.O Box 19676 00202

Nairobi, Kenya

Email: ckigondu@aar.co.ke

Signature: _____ Date: _____

3. DR. GONTIER CHARLES STUART BSc, MB;ChB, MMed

Lecturer,

Immunology Thematic Unit,

Department of Human Pathology,

School of Medicine,

University of Nairobi,

PO Box 19676-00202

Nairobi, Kenya

Email: christopher.gontier@uonbi.ac.ke

Signature: _____ Date: _____

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LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral therapy
CCC	Comprehensive care clinic
CD	Cluster of differentiation
CI	Confidence interval
CVL	Cervicovaginal lavage
cDNA	Complementary DNA
DMPA	Depomedroxyprogesterone acetate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACES	Family Aids Care and Education Services
GCLP	Good clinical laboratory practice
GUD	Genital ulcer disease
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HSV	Herpes simplex virus
IgG	Immunoglobulin G
IQR	Inter quartile range
ISID	International Society for Infectious Diseases
KNH	Kenyatta National Hospital
LGT	Lower genital tract
NKC	Natural killer cells

PI	Principal Investigator
PLWHA	People living with HIV and AIDs
RCTP	Research Care and Training Programme
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
RPM	Revolutions per minute
SANAS	South African National Accreditation System
SD	Standard deviation
SOP	Standard operating procedures
SPSS	Statistical package for social sciences
STD	Sexually transmitted disease
SSA	Sub Saharan Africa
TB	Tuberculosis
UNAIDS	United Nations Programme on HIV/AIDs
UNITID	University of Nairobi Institute of Tropical and Infectious Diseases
UON	University of Nairobi
WHO	World Health Organisation

ABSTRACT

BACKGROUND

Sub-Saharan Africa constitutes 65% of the global Human Immunodeficiency Virus and acquired immunodeficiency syndrome (HIV/AIDS) burden. Sixty percent of those infected are women. Contraceptives are very important in helping women make their reproductive health choices, the most commonly used in our set up being depomedroxyprogesterone (DMPA, which has been associated with increased viral loads in plasma and lower genital tract though contentious. Few studies have been done in this regard, however, no human studies have been done to compare HIV-1 plasma and lower genital tract viral loads in DMPA users with viral loads of those not on DMPA. Furthermore, since HSV-2 is a known risk factor for HIV, information on the effect of HSV-2 seropositivity on plasma and lower genital tract in relation to DMPA use is needed.

Objectives

To determine the effects of DMPA on both plasma and lower genital tract HIV 1 viral loads in seropositive ART naïve women attending a comprehensive care centre (CCC) in Kisumu, Kenya, and to examine the impact of HSV-2 seropositivity on the viral load concentrations..

Study design

This was a case control study.

Study area

This study was done using done using stored plasma and cervicovaginal lavage fluid samples obtained from participants in a previous study conducted in Kisumu, Kenya. The samples were transported to the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) laboratory for analysis.

Study population

Twenty one healthy cases of HIV-1 positive ART naïve women on DMPA and twenty one controls of healthy HIV-1 positive ART naïve women not on DMPA.

Materials and Methods

Plasma and cervicovaginal lavage fluid HIV-1 viral loads were determined using automated real-time reverse transcriptase polymerase chain reaction. Analysis was done using Stata v.12 for analysis to examine for association between DMPA and plasma and lower genital tract viral loads, association between plasma and lower genital tract viral loads and duration of DMPA use and for association between the viral loads with HSV-2 seropositivity.

Results

A total of 42 participants were included in the study, 21 cases and 21 controls. The mean age of cases was 26 years and 28 years for controls. Cases had higher mean plasma viral loads (Log_{10} 3.84) compared to controls (Log_{10} 3.78) though this was not statistically significant. There was a statistically significant positive correlation between plasma and lower genital tract viral loads in cases (r= 0.464) (p= 0.002) but no correlation in controls (r=0). Duration of DMPA use had no statistically significant effect on plasma and lower genital tract viral loads. HSV-2 seropositivity had no statistically significant effect on plasma and lower genital tract viral loads, in both cases and controls even after adjusting for confounding factors.

Conclusions

Use of DMPA was not associated with significant effects on concentrations of systemic and lower genital tract HIV-1 viral loads. There were no significant changes in the viral load concentration in cases and controls that were HSV-2 positive compared to those who were HSV-2 negative.

Recommendations

Larger more robust longitudinal studies are needed to clarify on the safety of DMPA in HIV positive women. In the meantime, use of dual contraception in HIV positive women are recommended.

INTRODUCTION

The Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organisation (WHO) estimates that 33.2million people are living with HIV/AIDs (PLWHA) globally, Sub-Saharan Africa constituting 65% of this burden.There has been an increase in the HIV prevalence in the recent years due to increased availability of highly active antiretroviral treatment (HAART) with reducing HIV associated mortalities. Globally, the incidence and HIV associated mortality rates however seem to have plateaued at 2.7million and 1.7million respectively, with sub-Saharan Africa making up 72% of each (1). Locally, however, the HIV prevalence has reduced from 14% in the mid-90s to 6.3% as at 2009 (2).This could be attributed to increased HIV/AIDs awareness and increased use of barrier methods to prevent transmission. African women have a disproportional HIV-1 infection burden, accounting for as many as 60%, through heterosexual transmission in over 90% of cases (1).

Injectable hormonal contraceptives are the most widely used form of contraception in Eastern and Southern Africa, and account for 40% of contraceptive use in these regions. This is largely due to increased availability and cost effectiveness. High unmet family planning need in Sub-Saharan Africa continues to be a major concern, with as little as 21% of women in the child bearing age (18-45years) being on contraceptives (3). In Kenya, 22% of the 40% of women in the childbearing age use injectable hormonal contraceptives. This is in sharp contrast to only 1.2% of these women using barrier methods despite the high HIV burden in the country. Nyanza province which has the highest HIV-1 burden in Kenya at 11% has a contraceptive prevalence rate of 37%, with 18% of these being injectable hormonal contraceptives**.** PLWHA mainly use injectable form of contraceptives (2) due to the aforementioned reasons. Approximately 20% of women at The RCTP/FACES in Lumumba Health Centre, CCC in Kisumu are using DMPA (4).

There is consensus on use of dual contraception, combining a hormonal method and barrier method, in HIV positive women. This is largely to enable women have control over their family planning needs and to mitigate against the potential risk of transmission of HIV to their partners (5). However, progestin based contraceptives (injectable DMPA and implantables) have been implicated in greater risk of HIV susceptibility and transmission among discordant couples (6). Although the mechanisms for this are less well understood, some studies have found alterations in the local and systemic immunity (7), higher rates of lower genital tract HIV

shedding and higher systemic viral loads in women using injectable hormonal contraceptives thus suggesting this as a possible mechanism for increasing HIV transmissibility (8). The higher plasma HIV viral load may also be associated with the risk of faster disease progression (9). The safety of progestin contraceptive use in HIV positive women therefore needs to be explored, with specific endpoints evaluating safety to the individual and transmissibility from these individuals.

Recent studies have also suggested that Human Herpes Simplex virus (HSV-2) infection may have a partial protective effect against sexual transmission of HIV through protection from bacterial STIs (10) but this is still highly debatable. Indeed, HIV negative, HSV positive DMPA users have been shown to have reduced susceptibility to HIV (11, 12). This observation is surprising since HSV-2 infection has also been shown to increase susceptibility to HIV infection (13). The present study was performed on samples collected in a previous study by Walong (2011), who found a high prevalence of HSV-2 seropositivity among the study population (7), 94% of the women on DMPA and 83% of women not using DMPA. The effect of HSV-2 on the plasma and genital viral loads was therefore found necessary in this study population.

Few studies exploring the effect of injectable hormonal contraceptives on HIV transmission with respect to viral loads have been done (6, 8, and 9). These studies have not compared the effect of the contraceptive on the viral loads with the viral loads of women not on DMPA. However, it can be postulated that DMPA may be associated with higher plasma HIV-1 viral loads which in turn may result in higher lower genital tract HIV-1 viral shedding, and hence increase in HIV transmission risk. Furthermore, it can be postulated that if the use of DMPA is associated with higher plasma HIV viral load, this may result in faster disease progression.

This study therefore aimed at exploring the risk of faster disease progression and increased HIV infection transmission associated with the use of DMPA by HIV positive women by simultaneously comparing both plasma and lower genital tract HIV viral loads among women on DMPA and those not on DMPA. The study also examined for an association between plasma and lower genital tract HIV viral loads with HSV-2 seropositivity.

LITERATURE REVIEW

DMPA USE AND HIV TRANSMISSION

The use of DMPA, a progestin based hormonal contraceptive has been associated with increased HIV-1 transmission although this is still inconclusive (7, 12, 14). Several mechanisms for this association have been proposed.

Firstly, DMPA use in seronegative women has been associated with increased HIV acquisition and whether or not this is significant remains an intense debate. A study conducted in Malawi in 2008 showed that use of injectables increased the risk of acquiring HIV through increasing rates of seroconversion by 10 fold after adjusting for other factors e.g. number of sexual partners (15). A similar study also showed increased seroconversion with DMPA (16). Morrison et al did a multistructural marginal analysis (MSM) on data from a previous study and found that hormonal contraceptives, specifically DMPA, in HSV-2 negative women were associated with increased HIV acquisition especially in the younger women aged between 18 and 24 years (12). A study by Baeten et al however showed that HIV acquisition in DMPA users was increased regardless of their HSV-2 status (17).

Secondly, progestins have been shown to cause hypoestrogenism resulting in changes in the vaginal milieu. This is evidenced by reduced hydrogen peroxide (H_2O_2) producing lactobacilli and reduced glycogen positive cell layers in the lower genital tract. Lactobacilli maintains the acidic pH in the vagina and are thought to directly kill free viruses in the lower genital tract and also prevent bacterial vaginosis and other lower genital tract infections which increase HIV acquisition. Miller et al also demonstrated that in addition, progestins cause thinning of the vaginal epithelium (14). Although this thinning is not as significant as shown in animal studies, (18) it is thought to contribute to increased acquisition and transmissibility of the infection due to reduced epithelial integrity (14).

Thirdly, progestins have been associated with alterations in local and systemic immunity. It is postulated that their use leads to polarization of the immune system towards a less robust inflammatory response as a result of reduced cytotoxic T cell production and activity and reduced proinflammatory cytokine production (7). They may also be responsible for higher CCR5 and CXCR4 rich mononuclear cells in the blood and lower genital tract hence increasing acquisition but this is still contentious (19).

Fourthly, DMPA use has been shown to increase risk of hyper infection with multiple HIV-1 genetic variants which eventually leads to faster disease progression hence increased transmissibility. Studies by Sagar et al showed that DMPA progestin is associated with multiple HIV-1 genetic variants and faster progression of HIV to AIDS and this has been implicated to increased transmissibility (20, 21).

Lastly, DMPA as will be discussed below, has been associated with changes in lower genital tract HIV viral shedding and plasma viral loads, these two being a major inference and pursuit in this study. Studies in this regard are scarce and results are largely conflicting.

DMPA USE AND SYSTEMIC HIV 1 VIRAL LOADS

A study by Lavreys and colleagues showed a significant increase in plasma RNA viral loads by a mean of +0.33 $\log_{10}$ copies/ml/month from a set point of 4.46$\log_{10}$ copies/ml ($p=0.03$) but no significance over time (>4months) in patients on DMPA (8). These high plasma viral loads at set point could make an important treatment target thus reducing disease transmission and progression (22). Studies have also shown that initiation of HAART earlier in the course of disease can reduce infectivity in discordant couples (23). In contrast, a 2010 case control study on 20 Chinese rhesus macaques showed no significant effect of DMPA on plasma viral loads and disease progression (24). Separate studies by Morrison et al and Richardson et al also showed no effect of DMPA on lower genital tract and plasma viral loads respectively hence no effect on transmission and disease progression (25, 26).This discrepancy in results has led to increased uncertainties in the effects and safety of DMPA in seropositive women and their partners.

DMPA USE AND LOWER GENITAL TRACT HIV 1 VIRAL LOADS

There is insufficient data regarding the effects of DMPA on lower genital tract HIV viral loads. A marginal structural model(MSM) analysis done on discordant couples by Heffron et al showed increased transmission from HIV-1 positive women on DMPA to their HIV negative male partners. This was linked to higher lower genital tract HIV-1 viral loads, especially greater in the younger women aged between 18 and 24 years. This study also showed that hormonal contraceptives could solely have a local effect on lower genital tract (LGT) viral loads without affecting the plasma viral loads (11).

A study conducted on HIV positive ART naïve women also showed an increase in genital viral load/viral shedding during luteal phase which corresponded to high progesterone levels.

Marginal significance on plasma viral loads was also noted. This study proposed an increase in the rate of sexual transmissibility as menses approached (27). In contrast,2 separate prospective cross sectional studies by Wang et al and Kovacs et al on larger cohorts, followed up for longer durations, showed no effect of DMPA and hormonal contraceptives respectively on lower genital tract shedding (28, 29).

USE OF LOWER GENITAL TRACT FLUID FOR VIRAL LOAD QUANTIFICATION

The importance of a normal lower genital tract is related to the risk of acquiring and transmitting HIV and other sexually transmitted diseases (STDs).This is highly dependent on pH, local immune factors, use of contraceptives and an individualôs sexual practices (30). Limited studies have been done on HIV-1 viral loads on other fluids other than plasma. Presence of HIV in lower genital tract fluids could make an important indirect marker of sexual transmissibility to partners and hence reduction in quantity of these viral loads together with plasma viral loads could therefore translate into some reduction in sexual transmission (31).

USE OF PLASMA FOR VIRAL LOAD QUANTIFICATION

Plasma is the most commonly used fluid in HIV viral load assays and is slowly becoming the cornerstone of monitoring disease progression and outcome and a marker of antiretroviral drug efficacy. This, especially in the developed world, is done together with CD4 cell counts (32). Although plasma HIV viral loads and CD 4 cell counts have been shown to improve monitoring of HIV progression and HAART response, plasma viral loads is a better predictor of progression to AIDS and death than CD4 cell counts (33).

USE OF REAL TIME REVERSE TRANSCRIPTASE PCR AS THE METHOD OF ANALYSIS

Real time reverse transcriptase PCR (real time RT-PCR) is a type of PCR used in molecular sciences. It begins with RNA reverse transcription into complementary DNA (cDNA) using reverse transcriptase enzyme. The resultant cDNA is then taken through several amplification cycles and the endpoint amplicons detected via use of fluorescent labelled probes attached to the amplified DNA. Its high sensitivity and specificity, approximated at close to 100%, detects low number of RNA copies. Other advantages include good precision and reproducibility, cost effectiveness, faster turnaround time and reduced chances of contamination. Rouet et al (2005) not only demonstrated this but also found an excellent correlation and concordance of results with other commercial kits (34).

CORRELATION BETWEEN HIV VIRAL LOADS IN PLASMA AND LOWER GENITAL TRACT

Only animal studies have correlated the plasma viral loads in DMPA treated animals with those of non DMPA treated animals (35, 36). There seems to be consensus however that a correlation exists between human plasma and lower genital tract viral loads in women not on hormonal contraceptives (25, 37, and 38). Few studies evaluating the correlation of plasma and lower genital tract viral loads in women on hormonal contraceptives have been done (11, 25). Heffron et al showed that DMPA had a probable local effect on the LGT viral loads leaving the plasma viral loads unaffected (11).This suggested that in DMPA users, high lower genital tract viral shedding does not necessarily reflect high plasma viral loads and vice-versa**.** However, a correlation of the lower genital tract and systemic viral loads in DMPA users with viral loads of subjects not on DMPA has not been done.

VALIDITY OF EVALUATING RNA IN STORED SPECIMENS

Analysis of biological markers, especially in research, may require testing of specimens that have been frozen for long periods with multiple rounds of thawing and freezing. There are concerns about thermal stability of the HIV-1 RNA in stored specimens. Global standardisation of sample storage to ensure minimal changes or none at all in the concentrations of the biological markers and accuracy of results has been set. The International Society for Biological and Environmental Repositories (ISBER) guidelines (2012) recommend storage of research plasma and cervical lavage fluid specimens for as long as 25 years in liquid nitrogen at sub-zero temperatures of up to -196Ŭ¢ with a maximum of 3 thawing and freezing episodes (39). This guidelines show that the lower the temperature, the lower the metabolic and degradation processes in the stored samples.

Separate studies by Ginnochio and Sebire et al showed that HIV-1 RNA in cell free plasma was stable for up to 6months and more than 12months respectively when stored at -70Ŭ¢ but both suggested further studies at lower temperatures (40, 41).

RATIONALE OF THE STUDY

It is postulated that HIV progression and outcome are largely affected by use of hormonal contraceptives in HIV positive women. Probably, this is achieved by these contraceptives by increasing HIV viral loads in both plasma and the lower genital tract. Since plasma and lower genital tract viral loads can be used as prime markers of HIV-1 disease outcome and transmission, respectively, one probable mechanism by which DMPA achieves this is by increasing these viral loads. However, this still remains a contentious issue. Few studies have evaluated the effects of DMPA on cervical and systemic HIV-1 viral loads. However, no studies have compared these viral loads in DMPA users with viral loads of women not on DMPA. It is conceptualised that studies in this group of women are necessary to possibly answer the above questions.

RESEARCH QUESTIONS

Is the use of DMPA associated with higher plasma viral loads in HIV positive ART naïve women which would imply a faster progression to AIDS? Is this higher plasma viral load associated with higher lower genital tract viral shedding which would imply increased risk of transmission?

And, is there any association between plasma and lower genital tract HIV viral loads and HSV-2 seropositivity?

HYPOTHESIS

The use of DMPA among HIV infected, ART naïve women is not associated with higher HIV-1 viral loads in plasma and lower genital tract.

STUDY OBJECTIVES

BROAD OBJECTIVE

To determine the effects of DMPA on HIV-1 plasma and lower genital tract viral loads in HIV positive ART naïve women attending RCTP/FACES CCC in Kisumu, Kenya.

SPECIFIC OBJECTIVES

1. To determine the effect of DMPA on plasma viral loads in HIV positive, ART naïve women on DMPA and compare these viral loads to viral loads of HIV positive ART naïve women not on DMPA.
2. To determine the effect of DMPA on lower genital tract HIV-1 viral loads in HIV positive, ART naïve women on DMPA and compare these viral loads to viral loads of HIV positive ART naïve women not on DMPA.
3. To determine the relationship between plasma and lower genital tract HIV-1 viral loads and the duration of DMPA use.

SECONDARY OBJECTIVE

To determine the impact of HSV-2 seropositivity on serum and lower genital tract HIV-1 viral loads.

STUDY DESIGN AND METHODOLOGY

STUDY DESIGN

This was a case control study using stored plasma and lower genital tract lavage specimens obtained from women who were part of another study, attending The Research Care and Training Program/Family Aids Care and Education Services (RCTP/FACES) comprehensive care clinic in Lumumba Health Centre, Kisumu, Kenya (7).

STUDY AREA

Samples were obtained from HIV positive ART naïve women attending RCTP/FACES comprehensive care clinic in Lumumba Health Centre, in Kisumu, Kenya between August 2010 and January 2011 and stored at -80ęC in a laboratory at the centre(7). These samples were transported to the University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) laboratory for analysis.

STUDY POPULATION

Study participants were part of another study and had consented to have their samples stored and used for future studies. They were HIV positive ART naïve women attending CCC at The Lumumba Health Centre in Kisumu, Kenya. The general inclusion criteria in that study (7) was women aged between 18-45 years who provided a written informed consent to participate in the previous and future studies. Their CD4 cell counts were above 350/uL on prior assessments

within the previous 3 months and they were asymptomatic for HIV/AIDS. These women were put into two categories namely cases and control. The cases were women on DMPA and who had used the contraceptive for at least 11completed weeks. The controls were women not on any hormonal contraceptive but with regular menstrual cycles.

The general exclusion criteria also from that study (7)was women below 18 and above 45 years of age, or on active treatment for TB or HIV, or who were acutely ill, or who had history of uterine cervical dysplasia, or had had hysterectomy, or were pregnant or lactating, or who reported absence of menses for over 60 days, or women with cervical inflammatory lesions including candidiasis, trichomoniasis, bacterial vaginosis, genital ulcer disease and gonococcal infections, or women who declined to give a written informed consent. The exclusion criteria for cases also included women not on DMPA, while the exclusion criteria for controls included women on other hormonal contraceptives.

SAMPLE SIZE CALCULATION

Sampsi Stata implementation formula was used to calculate the sample size using the mean endocervical concentrations of HIV-1 RNA in HIV-1 positive women on injectable contraceptive (11) as below.

Estimated sample size for two-sample comparison of means:

Test Ho: = m1 = m2 where m1 is mean of Controls & m2 is mean of Cases

$$n = \frac{\left(\frac{sd_1^2 + sd_2^2}{n_1 / n_2}\right)\left(Z_{1-\beta} - Z_{1-\alpha/2}\right)^2}{\left(m_1 - m_2\right)^2}$$

Assumptions:

Alpha = 0.05 (two-sided), power = 0.80, m1 = 3.14, m2 = 3.38, sd1 = .25, sd2 = .3, n2/n1 = 1.00

Estimated required sample sizes: n1 = 21, n2 = 21

SPECIMEN COLLECTION

This was done under a previous study by Walong et al between August 2010 and January 2011 (7). In that study, a physical and gynaecological examination was performed on each study participant. Specimen collection began with insertion of a sterile speculum and CVL fluid collected as follows: the cervix was bathed with 10 ml of sterile saline which was allowed to pool in the posterior fornix.

The fluid was then aspirated using a plastic bulb pipette and dispensed into a sterile container. Cells within the CVL fluid were separated by centrifugation at 1000 rpm for 10 minutes at 4^{o}C. The following samples in 1ml aliquots were then stored at -80^{o} C for mucosal immunity analysis: one with the cell pellet & 9 with supernatant fluid. Cervical and vaginal swabs from all potential study participants were screened for certain sexually transmitted infections namely *Neisseria gonorrhoea,* agents of bacterial vaginosis and *Trichomonas vaginalis.*Blood was also collected in EDTA and plain bottles. These were used for total lymphocyte, CD 4 cell count and HSV-2 IgG analysis. The latter was to allow for subgroup analysis of plasma and lower genital viral load in relation to HSV-2 seropositivity. Plasma was also stored at -80^{o}C for future studies in mucosal immunity in laboratories at The Research Care and Training Program/Family Aids Care and Education Services (RCTP/FACES) comprehensive care clinic in Lumumba Health Centre, Kisumu, Kenya (7).

SPECIMEN ANALYSIS

For this study, the Principal Investigator was involved in specimen identification, entering specimen details into the questionnaire, thawing of the frozen stored specimens, pipetting, machine calibration and running the specimens together with the positive and the negative controls as per the Standard Operating Procedure (SOP).

Analysis of the specimens was done at the University Of Nairobi Institute Of Tropical and Infectious Diseases (UNITID) laboratory using the M2000rt automated molecular machine and Abbott reverse transcriptase and amplification PCR kits.

Briefly, reagents and specimens were prepared at the pre-amplification RNA extraction area. This began with thawing of samples, internal controls and standards at 15-30ę. Each was then thoroughly mixed 3 times for 2-3seconds using a vortex mixer. Three bottles of HIV-1 lysis agent were prepared of which each bottle supported a batch of maximum 24 specimens. 5ml reaction tubes were placed in a Micro Amp tray locked in place with retainer and 2.5ml of the prepared HIV lysis agent and 100ul of magnetic Iron particles added into each tube. 1ml

sample was then added into each of these tubes and mixture thoroughly mixed. This mixture was then incubated at 50ęfor 20 minutes after which the lysate was removed from each tube without disturbing the captured magnetic particles. The captured particles were then taken through 3 washes. Elution buffer was added to the captured particles. A master mix was then prepared by adding 270ul of HIV-1 activation reagent and 950ul of oligonucleotides reagents into the thermostable rTth DNA polymerase bottle. Measures of 50ul of the sample elute and 50ul of the amplification master mix were then mixed in the 96-well reaction plates, which were then sealed with an Abbott m2000rt adhesive cover and transferred to the m2000rt machine. Here, automated reverse transcription, amplification and detection through fluorescent labelled DNA probes were done. These last 3 procedures were repeated for 35-45 cycles each at different temperatures and timings. The results were automatically generated by the machine and interpreted as: not detected (below assay threshold), detected (<40 copies/ml), actual copies of 40 to 10,000,000 copies/ml and >10,000,000 copies/ml. (Appendix IV).

HSV-2 data was obtained from the previous analysis conducted on the samples as previously described (**7**). Briefly, HSV-2 IgG was assayed using HSV-2 IgG specific enzyme linked immunoassay kit by Kalon diagnostics, United Kingdom and optic densities above a cut off point, determined from positive and negative controls, were reported as positive.

QUALITY ASSURANCE

Safety practices were adhered to during specimen collection in the previous study and this was done under sterile conditions. The specimens were stored at -80ęC at The SANAS, GCLP accredited laboratories at The RCTP/FACES in Lumumba health centre in Kisumu, Kenya. Stringent daily automated temperature charting, recording and manual archiving was ensured for maximal preservation of the samples. The laboratories had a standby functional generator and an uninterruptible power source (UPS) in place in case of electricity blackouts. Transportation of the samples was done with a cold chain transport courier. The samples were then stored at The University of Nairobi Institute of Tropical and Infectious Diseases (UNITID) laboratory at -80ęc. Prior to analysis, all bench surfaces were cleaned with bleach (10% Jik). During analysis, strict adherence to standard operating procedure (SOP), mainly to avoid contamination, was adhered to. This included use of appropriate protective gear and preparation of reagents and patient samples accordingly as outlined in the SOP. Effectiveness of the analytical procedure was further tested by running internal quality control negative samples (undetectable levels) and positive quality control samples (low positive 145-4,511 copies/ml and high positive 15,488-489,779 copies/ml) with each test to evaluate run validity

and through use of a well calibrated machine. Decontamination of infectious surfaces using 10% Jik and safe disposal of consumables was done after analysis. The remaining specimens were stored at The UNITID laboratories at -80ǁ¢ for future use in other studies.

ETHICAL CONSIDERATION

Approval of the study was obtained from The Kenyatta National Hospital/University of Nairobi Ethics and Research Committee (KNH/UON ERC) prior to commencement (Appendix V).

A written informed consent was obtained from the study participants in the previous study included storage and use of their data and samples for future analysis/studies (Appendix III). Data from the patients will be kept confidential.

DATA ANALYSIS

The data collected included independent variables that included age, marital status, level of education and occupation of participants. These were collected using a standard questionnaire (Appendix I). Continous data included the obtained lower genital tract and plasma viral loads for cases and controls. Categorical data included participants on DMPA and non- DMPA users. HSV-2 categorical data was obtained from results of the previous study (7). The data was entered into Statistical Package for Social Sciences version 17 (SPSS v. 17). The viral loads were converted to Log_{10} for data analysis.

Analysis of the descriptive, univariate, bivariate and multivariate statistical analyses was done using Stata v.12 (Stata Corp, College Station Texas).

Continuous data (viral loads) was presented descriptively in form of means (standard deviations [SD]) or medians (inter-quartile range [IQR]) as appropriate. Frequency tables (%) were used for categorical variables. Pictorial presentation using histograms and scatter plots was done as appropriate.

Bivariate analysis was done using t-test or Mann Whitney-U/Rank sum test as appropriate for continuous variables and fisherôs exact test for categorical variables to determine presence of statistically significant association between DMPA use, participant characteristics and outcome variables (plasma and lower genital tract viral loads) and reported as descriptive statistics as well as the respective p values.

Univariate and multivariate linear regression *(ANOVA)* analyses were used to assess the relationship between the plasma or lower genital tract viral loads, DMPA use and participant

characteristics. The same analyses were done to assess for the effects of duration of DMPA use on the plasma and lower genital tract HIV-1 viral loads and HSV-2 seropositivity. The regression coefficients, corresponding 95% confidence intervals and respective p values were report for each of the univariate and multivariate models and p value of <0.05 considered statistically significant.

RESULTS

The study was conducted from June 2012 to March 2013. A total of 42 participants were included in the study, 21 cases and 21 controls.

Table 1 shows the characteristics of the study population .The women on DMPA were significantly younger than the women not on DMPA, median age in years was 26(IQR: 23 - 30) vs. 28(IQR: 25 - 34), (Mann Whitney-U test, p= 0.016. Generally, the women on DMPA were younger with a narrow age distribution where as those not on DMPA were older with a more dispersed age distribution. The overall majority of the women were married 25(59.5%) or single 9(21.4%). However, there was no significant statistical difference in marital status between the two groups (Fishers test, p=1.000). Most of the subjects 30(71.4%) had primary education as the highest level of education constituted by 16(76.2%) cases and 14(66.7%) controls but without any significant statistical difference (Fishers test, p=0.485).

Table 1 Socio-demographic variables in cases and controls

Variables	Controls (No DMPA)	Cases (DMPA)	Total	P value
Age of participant in years *+				
Median (IQR)	28(IQR: 25 - 34)	26(IQR: 23-30)		0.016
Marital status n (%) **				1.000
Single	5 (23.8)	4 (19)	9 (21.4)	
Married	12 (57.1)	13 (61.9)	25 (59.5)	
Separated	2 (9.5)	3 (14.3)	5 (11.9)	
Divorced	1 (4.8)	0 (0)	1 (2.4)	
Widowed	1 (4.8)	1 (4.8)	2 (4.8)	
Highest level of education n (%) **				0.485
Primary	14 (66.7)	16 (76.2)	30 (71.4)	
Secondary	4 (19)	5 (23.8)	9 (21.4)	
College	2 (9.5)	0 (0)	2 (4.8)	
University	1 (4.8)	0 (0)	1 (2.4)	
Occupation n (%) **				0.032*

Housewife	2 (9.5)	1 (4.8)	3 (7.1)
Farmer	3 (14.3)	0 (0)	3 (7.1)
Unemployed	1 (4.8)	6 (28.6)	7 (16.7)
Professional	3 (14.3)	0 (0)	3 (7.1)
Other	12 (57.1)	14 (66.7)	26 (61.9)

**** Statistically significant (p<0.05)***

***^ t-test, ** Fishers test, *+ Mann Whitney-U test**

On bivariate analysis, there was no significant statistical difference in the prevalence of HSV2 IgG positives among the cases and controls, 20(95.2%) vs. 18(85.7%); (Fishers test, p=0.606).There was also no significant statistical difference in the mean plasma viral load copies per mL (log base 10) among the controls and cases 3.78(0.81) vs. 3.84(0.76) respectively; (t-test, p=0.7969) .The median cervical viral load copies per mL (log base 10) among the cases was higher than among the controls 1.30(0, 2.21) vs. 0 (0, 0) respectively. These changes, however, did not reach significant statistical difference (Mann Whitney U-test, p=0.0814) (Table 2, Figure 1 and Figure 2).

Table 2 Bivariate analysis of plasma and lower genital tract HIV-1 viral loads, HSV-2 seropositivity and duration of DMPA use in cases and controls.

Variables	**Controls (No DMPA)**	**Cases DMPA)**	**Total**	**P value**
Duration on DMPA (Months)				
Median (IQR)	n/a	12 (10,24)	12 (10,24)	n/a
HSV2 IgG n (%) **				
Positive	18 (85.7)	20 (95.2)	38 (90.5)	0.606
Plasma viral load(log_{10}) *^				
mean(SD)	3.78 (0.81)	3.84 (0.76)	3.81 (0.77)	0.797
Cervical viral load(Log_{10}) *+				
median(IQR)	0 (0,0)	1.30 (0,2.21)	0 (0,1.86)	0.081

**** Statistically significant (p<0.05)***

***^ t-test, ** Fishers test, *+ Mann Whitney-U test**

Figure 1 Histogram showing the distribution of plasma HIV-1 viral loads in cases and controls (Log_{10})

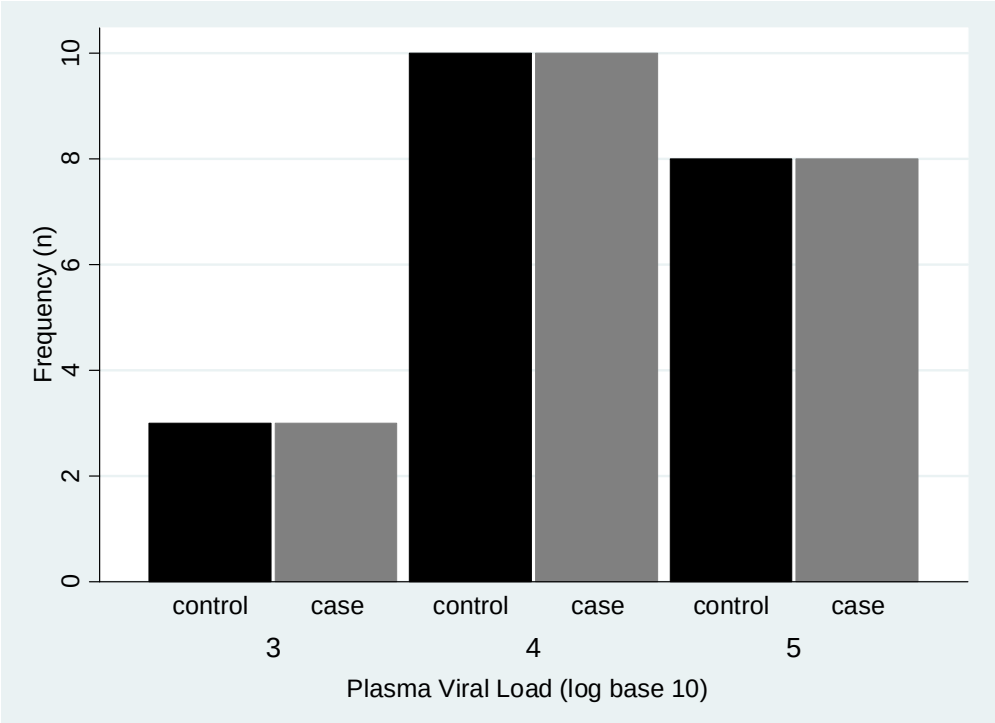
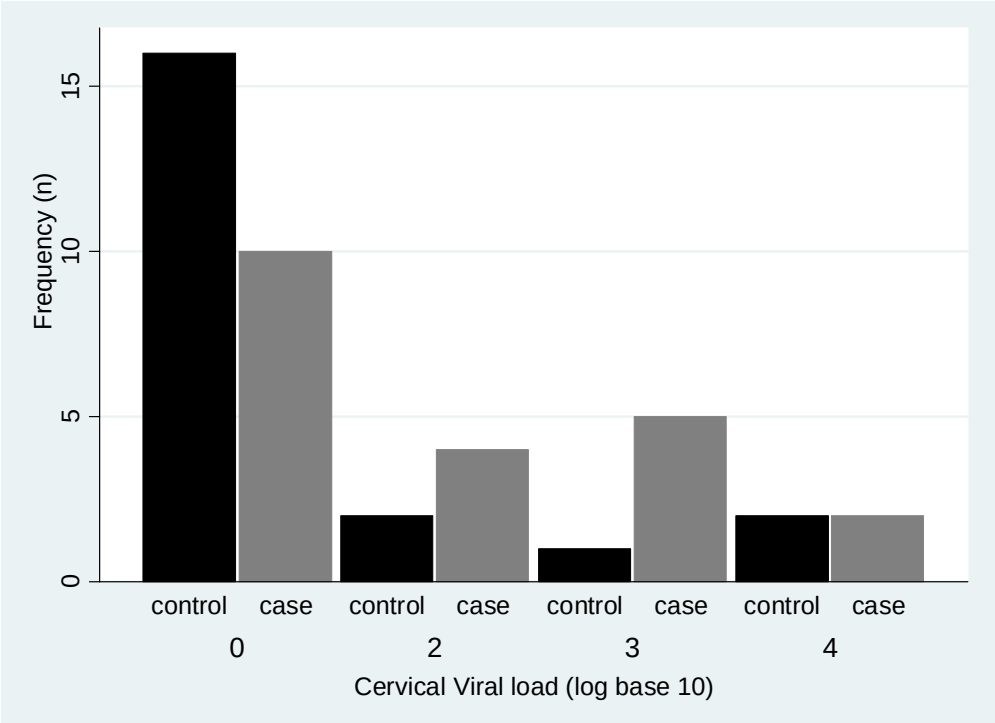


Figure 2 Histogram showing the distribution of lower genital tract HIV-1 viral loads in cases and controls (Log_{10})



PLASMA HIV-1 VIRAL LOADS IN RELATION TO DMPA USE

Using univariate linear regression analysis, the women on DMPA had a slightly higher plasma viral load copies /mL (log base 10) than the women not on DMPA. However, the difference in the plasma viral load between the two groups was not statistically significant, 0.063 (95% CI -0.426 0.551); p = 0.797. When the plasma viral load between the cases and controls was adjusted for age in multivariate analysis, the women on DMPA still had a higher plasma viral load than those not on DMPA. Similarly, this difference was not statistically significant, 0.020 (95% CI -0.026 - 0.063); p= 0.364 (Table 3).

Table 3 Regression analysis of plasma HIV-1 viral loads in relation to DMPA use (n=42)

Outcome: Plasma viral loads (Log_{10})	Mean viral loads	Univariate Coefficient 95% CI	P value	Multivariate coefficient 95% CI	P value
Controls (n=21)	3.78	Reference			
Cases (n=21)	3.84	0.063(-0.426 -0.551)	0.797	0.020(-0.026-0.063)	0.364
Age (Years)		-0.015(-0.025-0.054)	0.454	0.154(-0.375-0.684)	0.559

LOWER GENITAL TRACT HIV-1 SHEDDING IN RELATION TO DMPA USE (N=42)

As shown on table 4 below, the women on DMPA had a higher cervical viral load copies /mL (log base 10) than the women not on DMPA in univariate linear regression analysis. However, this difference was not statistically significant, 0.597 (95% CI -0.167 - 1.361); p = 0.122. When the lower genital tract HIV-1 shedding was adjusted for plasma viral load and age in multivariate analysis, women on DMPA still had higher cervical viral load copies/mL compared to the women not on DMPA. Similarly, this difference was not statistically significant, 0.497 (95% CI -0.316 - 1.310); p= 0.224.

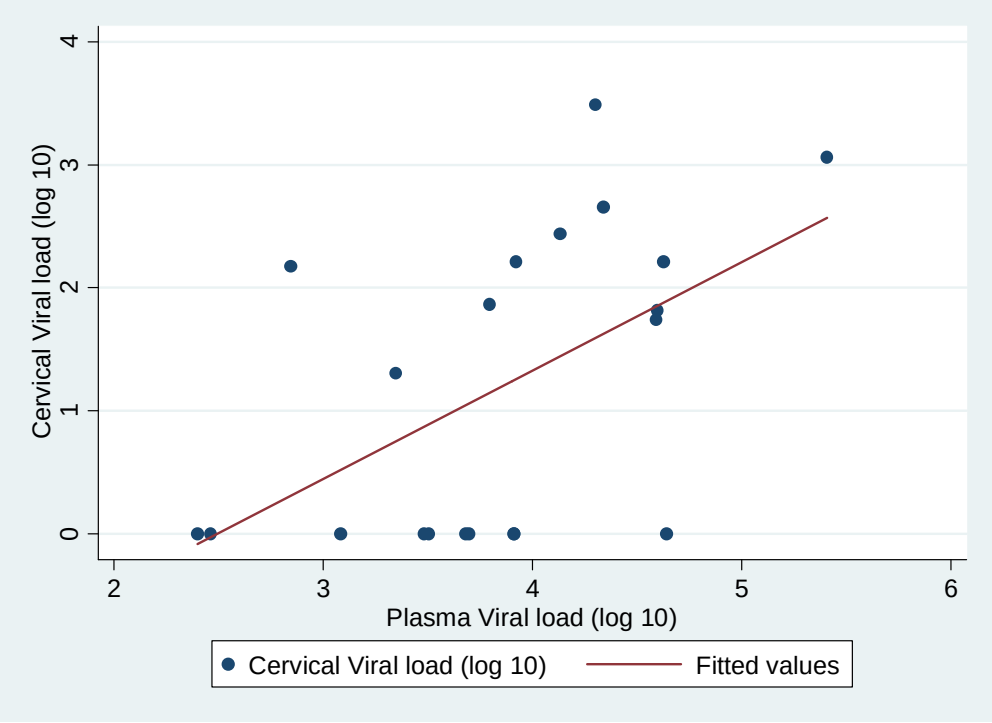
Table 4 Regression analysis of lower genital tract HIV-1 viral shedding in relation to DMPA use (n=42)

Outcome: LGT viral loads (Log_{10})	Median viral loads	Univariate Coefficient 95% CI	P value	Multivariate coefficient 95% CI	P value
Controls (n=21)	0	Reference			
Cases (n=21)	1.30	0.597(-0.167-1.361)	0.122	0.497(-0.316-1.310)	0.224
Plasma viral loads(Log_{10})		0.475(-0.017-0.967)	0.058	0.476(-0.019-0.972)	0.059
Age (years)		-0.023(-0.087-0.040)	0.462	-0.015(-0.081-0.052)	0.652)

CORRELATION OF PLASMA AND LOWER GENITAL TRACT VIRAL LOADS

The scatter plot below represents a weak positive correlation between plasma and lower genital tract viral loads in cases i.e. the higher the plasma viral loads the higher the lower genital tract viral loads (figure 3). Using descriptive trends, the mean ratio of plasma to lower genital tract viral loads, in subjects that had detectable lower genital tract viral loads, was two (2) as described in Table 5, indicating that the subjects had twice as high plasma viral loads compared to lower genital tract viral loads.

Figure 3 Scatter plot showing the correlation of plasma and lower genital tract HIV-1 viral loads in cases (Log_{10})



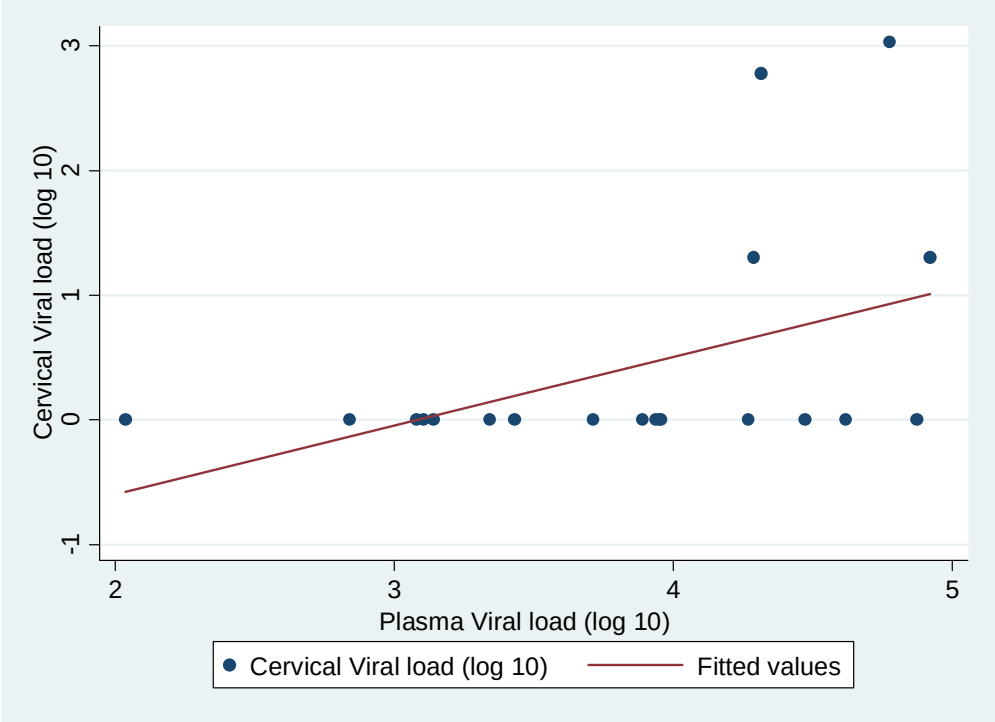
Correlation coefficient (r) =0.4644

Table 5 Trend of plasma and lower genital tract viral loads in cases who had detectable lower genital tract viral loads

N = 12	**Plasma viral loads (Log10)**	**LGT viral loads(Log10)**	**Plasma: LGT viral load**
Case 10	3.9	2.2	1.8
Case 11	3.9	1.4	2.7
Case 12	3.9	1.8	2.2
Case 13	4.0	2.5	1.6
Case 14	4.3	2.3	1.9
Case 15	4.4	2.6	1.7
Case 16	4.6	2.8	1.6
Case 17	4.6	3.7	1.2
Case 18	4.7	2.3	2.0
Case 19	4.9	1.8	2.7
Case 20	4.9	1.7	2.9
Case 21	5.4	3.1	1.7
Mean ratio			**2.0**

The scatter plot below shows that there was no correlation between plasma and lower genital tract viral loads among the controls (figure 4).

Figure 4 Scatter plot showing the correlation of plasma and lower genital tract viral loads in controls (Log_{10})



Correlation coefficient (r=0)

PLASMA HIV-1 VIRAL LOAD AND DURATION OF DMPA USE

In univariate linear regression analysis, women who had used DMPA for more than 12 months had higher plasma viral load copies /mL (log base 10) than women who had for used DMPA for 12 months or less. However, this difference was not statistically significant, 0.233 (95% CI -0.512 - 0.979); p = 0.520. When the plasma viral load was adjusted for age in multivariate analysis, women who had used DMPA for more than 12 months still had higher plasma viral load than women who had used DMPA for 12 months or less. Similarly, this difference was not statistically significant, 0.221 (95% CI -0.566 - 1.008); p= 0.562 (Table 6).

Table 6 Regression analysis of plasma HIV-1 viral loads in relation to duration of DMPA use (n=21)

Outcome: Plasma viral loads (Log_{10})	Mean viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
DMPA use <12mths(n=14)	3.76	Reference			
DMPA use >12mths (n=7)	3.99	0.233(-0.512-0.979)	0.520	0.221(-0.556-1.008)	0.562
Age (years)		-0.015(-0.025-0.054)	0.454	0.007(-0.086-0.098)	0.885

LOWER GENITAL TRACT HIV-1 VIRAL LOAD AND DURATION OF DMPA USE

Table 7 shows the relationship between lower genital tract HIV-1 viral loads and the duration of DMPA use. In univariate analysis, women who had used DMPA for more than 12 months had a higher cervical viral load copies /mL (log base 10) than women who had used DMPA for 12 months or less. However, this difference was not statistically significant, 0.601 (95% CI -0.600 1.803); p = 0.308. When the cervical viral load was adjusted for plasma viral load copies /mL (log base 10) and age in multivariate analysis, there was still a higher cervical viral load shedding among women who had used DMPA for more than 12 months than among those who had used DMPA for 12 months or less. However, this difference was also not statistically significant, 0.432 (95% CI -0.695 - 1.559); p= 0.430).

Table 7 Regression analysis of lower genital tract HIV-1 viral loads in relation to duration of DMPA use (n=21)

Outcome: LGT viral loads (Log_{10})	Median viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
DMPA use <12mths(n=14)	0.08	Reference			
DMPA use >12mths (n=7)	1.58	0.601(-0.600-1.803)	0.308	0.432(-0.695-1.559)	0.430
Plasma viral loads(Log_{10})		0.475(-0.017-0.967)	0.058	0.845(0.142-1.547)	0.021
Age (years)		-0.023(-0.087-0.040)	0.462	-0.015(-0.145-0.116)	0.814

PLASMA HIV-1 VIRAL LOAD IN CASES IN RELATION TO HSV2 SEROPOSITIVITY

Table 8 shows the relationship between plasma HIV-1 viral loads and HSV-2 seropositivity in DMPA users. In univariate analysis, HSV-2 positive women on DMPA had higher plasma viral load copies /mL (log base 10) than DMPA users who were HSV-2 negative. However, this difference was not statistically significant, 0.354(95% CI -1.306-2.014); p = 0.660. When the plasma viral load was adjusted for age in multivariate analysis, there was still a higher plasma viral load among HSV-2 positive women on DMPA compared to the HSV-2 negative cases. However, this difference was also not statistically significant, 0.370 (95% CI -1.341-2.080); p= 0.655.

Table 8 Regression analysis of plasma HIV-1 viral loads in cases in relation to HSV-2 seropositivity (n=21)

Outcome: Plasma viral loads (Log_{10})	Mean viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
HSV2 IgG negative (n=1)	3.50	Reference			
HSV2 IgG positive (n=20)	3.86	0.354(-1.306-2.014)	0.660	0.370(-1.341-2.080)	0.655
Age (years)		0.012(-0.076-1.000)	0.777	0.013(-0.077-0.104)	0.761

PLASMA HIV-1 VIRAL LOAD IN CONTROLS IN RELATION TO HSV-2 SEROPOSITIVITY

Table 9 shows the relationship between plasma HIV-1 viral loads and HSV-2 seropositivity in women not on DMPA (controls). In univariate analysis, HSV-2 positive controls had higher plasma viral load copies /mL (log base 10) than HSV-2 negative controls. However, this difference did not reach statistical significance, 0.408(95% CI (-0.696-1.512); p = 0.448. When the plasma viral load was adjusted for age in multivariate analysis, there was still a higher plasma viral load among HSV-2 positive controls compared to the HSV-2 negative controls. However, this difference was also not statistically significant, 0.370 (95% CI -1.341-2.080); p= 0.655.

Table 9 Regression analysis of plasma HIV-1 viral loads in controls in relation to HSV-2 seropositivity (n=21)

Outcome: Plasma viral loads (Log_{10})	Median viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
HSV2 IgG negative (n=3)	3.36	Reference			
HSV2 IgG positive (n=18)	3.84	0.408(-0.696-1.512)	0.448	0.370(-1.341-2.080)	0.655
Age (years)		0.022(-0.031-0.076)	0.397	0.017(-0.038-0.073)	0.520

LOWER GENITAL TRACT HIV-1 VIRAL LOAD IN CASES IN RELATION TO HSV-2 SEROPOSITIVITY

In univariate linear regression analysis, HSV-2 positive women on DMPA had higher lower genital tract viral shedding copies /mL (log base 10) than cases that were HSV-2 negative. However, this difference was not statistically significant, 1.249 (95% CI -1.420-3.917); p = 0.340. When the lower genital tract viral load was adjusted for plasma viral loads and age in multivariate analysis, HSV-2 positive cases still had higher lower genital tract viral load than HSV-2 negative cases. Similarly, this difference was not statistically significant, 0.833 (95% CI -0.461-2.128); p= 0.200 (Table 10).

Table 10 Regression analysis of lower genital tract HIV-1 viral loads in cases in relation to HSV-2 seropositivity (n=21)

Outcome: LGT viral loads (Log_{10})	Median viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
HSV2 IgG negative(n=1)	0	Reference			
HSV2 IgG positive(n=20)	0.124	1.249(-1.420-3.917)	0.340	0.833(-0.461-2.128)	0.200
Plasma viral loads(Log_{10})		0.475(-.0.017-0.967)	0.058	0.451(-0.048-0.950)	0.075
Age (years)		-0.006(-0.138-0.151)	0.927	-0.033(-0.095-0.028)	0.280

LOWER GENITAL TRACT HIV-1 VIRAL LOAD IN CONTROLS IN RELATION TO HSV-2 SEROPOSITIVITY

In univariate linear regression analysis, HSV-2 positive HIV positive women not on DMPA had higher lower genital tract viral shedding copies /mL (log base 10) than controls that were HSV-2 negative. However, this difference was not statistically significant, 0.691 (95% CI-0.891-2.272); p = 0.372. When the lower genital tract viral load was adjusted for plasma viral loads and age in multivariate analysis, HSV-2 positive controls still had higher lower genital tract viral load than HSV-2 negative controls. Similarly, this difference was not statistically significant, 0.945 (95% CI-1.483-3.374); p= 0.423 (Table 11).

Table 11 Regression analysis of lower genital tract HIV-1 viral loads in controls in relation to HSV-2 seropositivity (n=21)

Outcome: LGT viral loads (Log_{10})	Median viral loads	Univariate Coefficient 95% CI	P value	Multivariate Coefficient 95% CI	P value
HSV2 IgG negative(n=1)	0	Reference			
HSV2 IgG positive(n=20)	0.60	0.691(-0.891-2.272)	0.372	0.945(-1.483-3.374)	0.423
Plasma viral loads(Log_{10})		0.880(0.217-1.544)	0.012	0.451(0.154-1.552)	0.020
Age (years)		-0.009(-0.091-0.072)	0.803	-0.001(-0.129-0.127)	0.280

DISCUSSION

It has been previously hypothesised that women on DMPA would have higher plasma viral loads than those not on DMPA. This hypothesis is based on case control animal studies that have found higher viral loads in DMPA injected animals compared to the controls (34, 35). However, a more recent case control animal study has found contrasting results (24).The present study did not find a significant effect of DMPA on plasma viral loads even after adjusting for confounding factors. Although Cejtin et al (42) had similar findings, their study looked at the effect of hormonal contraceptives in general and included both injectable and oral hormonal contraceptives unlike in the present study where only the effect of DMPA on plasma viral load was examined. Watts et al (43) also did not find any association between DMPA and plasma viral load in a short longitudinal study. In that study, all the women were on ART compared to the present study where all the women were ART naïve. However, the present finding contrasts with those by Lavreys et al (8), a prospective study, where newly HIV infected women on DMPA were found to have a higher viral set-point compared to the women not on DMPA. Multivariate analysis in that study, controlled for sexual behaviour, use of DMPA at the time of HIV-1 infection was associated with a higher viral set point, compared to women not on DMPA. This effect was specific to DMPA use and was not seen in women who used oral contraceptive pills. The authors speculated that there may be an interaction between DMPA use and virus-host dynamics established during the early phase of infection. Nevertheless, the change in HIV RNA levels over time did not differ between women on DMPA and those not on hormonal contraceptives. Thus, although theoretically, DMPA could have an effect on viral set point if present at the time of infection because of effects on susceptibility of the genital tract to HIV yielding more infected cells or on early events in viral replication, DMPA does not appear to cause an increase in HIV RNA levels among women with established infection (42)

This study found that there was an overall higher HIV viral load in the lower genital tract among the DMPA users than the non-DMPA users both in univariate and multivariate analysis. However, this difference was not statistically significant. Although not statistically significant, this could still be responsible for an increase in infectivity among these individuals therefore justifying use of dual contraception where barrier methods and DMPA are used concurrently (5). It has been suggested that use of hormonal contraceptives can affect genital tract HIV-1 RNA shedding by influencing selected cytokine production in the genital tract, which may modulate the replication of HIV-1 (44). Furthermore, the observation that genital tract HIV-1

RNA shedding fluctuates with the menstrual cycle also suggests that there may be a hormonal influence on HIV expression (27). There is scarcity of data on the effects of DMPA on lower genital tract viral loads and most of the studies done in this regard have looked at effect of DMPA HIV-1 DNA shedding (45, 46) instead of HIV-1 RNA like in this study. Although previous studies conducted in populations of African women have shown the use of hormonal contraceptives to be associated with increased pro-viral HIV-1 DNA cervical shedding (28, 44), no association between hormonal contraception use and genital tract HIV-1 RNA shedding has been found. It can be speculated therefore that this pro-viral DNA in the cells is released into circulation and other compartments leading to high lower genital tract HIV-1 RNA viral shedding. In a study conducted among American women, no association was found between hormonal contraception use and HIV-1 RNA genital tract shedding, even after adjusting for plasma HIV-1 RNA levels, sexually transmitted infection status, CD4 cell count and age (47).

Factors influencing genital HIV viral shedding may be hypothesised to be doing this by influencing plasma viral loads which may then have a direct relationship with the genital viral load. It is thus of importance that studies looking at the relationship of DMPA on genital viral shedding examine for the influence on plasma viral load. Although some studies have found a correlation between plasma HIV viral load and genital viral shedding, this has not always been the case. The phenomenon of genital tract HIV-1 RNA shedding even among women with non-detectable HIV-1 RNA plasma levels on ARV treatment has previously been described (29,46). This study correlated plasma and lower genital tract viral loads in DMPA users and plasma and lower genital tract viral loads in non-DMPA users. There was a weak positive correlation in DMPA users ($r=0.464$) but no correlation in non-DMPA users ($r=0$). The former showed a mean ratio of 2 between plasma and lower genital tract viral loads in subjects that had detectable lower genital tract viral loads. The weak correlation could have been contributed by the small sample size. In cases, therefore, the higher plasma viral loads the higher lower genital tract viral loads. This finding is in agreement with several other studies in this regard (25, 29, 36, 46, 48, and 49). A study by Wagner et al demonstrated that a higher plasma viral load was associated with higher transudation of the virus into other compartments such as the lower genital tract (50). However, a study by Rasheed et al (1996) disputed this finding and suggested that plasma and lower genital tract viral loads are two independent, unrelated events. This study suggested that only local factors e.g. trauma, infections, cervical ectopy and abrasions of the lower genital tract are responsible for alterations in lower genital tract HIV-1 viral loads (51).

If it is hypothesized that DMPA use is associated with an increase in plasma and lower genital tract HIV-1 viral load, it can be expected that there would be a correlation between duration of DMPA use and plasma viral load. However, the present study found no statistically significant correlation between duration of DMPA use and levels of plasma and lower genital tract viral loads. Generally, women who had used DMPA for over 12 months had higher plasma and lower genital tract viral loads than women who had used the contraceptive for 12months or less, but this difference was not statistically significant. The higher, although not significant, lower genital tract viral loads with time may be attributed to the observation that women on DMPA have a time dependent reduction in the IL-1ß and an increase in IL-10 levels in cervico-vaginal lavage leading to reduced proinflammatory response and polarisation to a less robust humoral immune activity, respectively (7). On the other hand, contrasting studies have shown that DMPA used for shorter durations (4-6 months) leads to significantly higher viral loads at set point in both plasma and lower genital tract with tapering levels thereafter(8,25). This increase in viral loads at set point in women on DMPA can be used as a measure of virulence and an important marker of the hostôs ability to contain the virus after infection (52, 53). Further studies are, however, necessary.

HSV has been found to up-regulate HIV-1 replication in cell-culture studies (54, 55). Furthermore, studies of chronic HIV-1 infection have shown a link between HSV infection and increased HIV replication (56, 57). HSV-2 has also been shown to increase the levels of natural killer cells (NKCs) in the blood which in return activate CD4 lymphocytes by presenting the HSV-2 antigen to the lymphocytes. This increase in CD4 lymphocytes can be speculated to lead to an increase in HIV-1 binding sites with an eventual increase in HIV-1 viral loads in plasma (58). On the other hand, HSV-2 negative women on DMPA have been shown to have an increased risk of HIV- 1 acquisition and whether this leads to an effect in systemic viral loads and faster disease progression is still unknown (12). Thus, an association between the presence of HSV-2 and plasma and lower genital HIV viral load can be expected. Despite the high prevalence of HSV-2 seropositivity among study subjects, this study found no significant effect of HSV-2 on plasma and lower genital tract viral loads in both cases and controls even after adjusting for confounding factors. The lack of significant rise in viral loads could be attributed to the fact that most women, both cases and controls, were HSV-2 positive (cases 94%, controls 83%). This HSV-2 positivity could have led to higher lower genital tract cytokine concentrations leading to better local and systemic activation of the innate and

humoral immunity against the HIV(7). This is however debatable. There is paucity of data evaluating the effect of HSV-2 seropositivity on HIV-1 viral loads in women on DMPA co-infected with HIV-1. Lavreys et al, however, found that the presence of genital urinary disease (GUD) during the early phase of HIV-1 infection was associated with a more rapid increase in plasma viral load during chronic HIV-1 disease (8). The authors suggested the possibility that the effect of GUD on plasma viral load could also be due to more-frequent HSV reactivation in the course of HIV-1 infection. Furthermore, when they adjusted their analysis for genital ulcers that occurred later during the course of the disease, the association between change in virus load over time and GUD near the time of infection remained. Majority of the GUD in their study were clinically diagnosed as episodes of genital herpes (8).

CONCLUSION AND RECOMMENDATIONS

CONCLUSION

i. Use of DMPA was not associated with significant effects on systemic and lower genital tract HIV-1 viral load concentration.
ii. The study found no effect of duration of DMPA use on lower genital tract and systemic viral load concentration.
iii. A positive correlation between systemic and lower genital tract viral load was apparent in cases (HIV positive, ART naive women on DMPA).
iv. There was no statistically significant difference in the systemic and lower genital tract viral load concentration in cases and controls that were HSV-2 positive compared to those who were HSV-2 negative.

RECOMMENDATIONS

1. This study showed slight but insignificant higher plasma and lower genital tract HIV-1 viral loads in women on DMPA. Since higher HIV-1 viral loads in plasma and genital tract are associated with faster progression to AIDS, and increased transmission rates of HIV, respectively, larger more robust studies are needed to clarify on the role of DMPA in this regard.

LIMITATIONS

1. Since the specimens collected were single point samples, longitudinal studies are needed to examine for the effect of DMPA on plasma and lower genital tract HIV-1 viral load over time.
2. It would have been useful to include cytological changes in the vaginal and cervical smears and correlated the findings with viral load and HSV status but due to finances and time limitations this was not done.

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APPENDICES

APPENDIX I: STUDY QUESTIONNAIRE

SECTION 1 – SOCIODEMOGRAPHIC DATA

STUDY NO : PREVIOUS STUDY _____ CURRENT STUDY _____

CCC ID : _____

DATE : _____

AGE (tick one)

1. 18-20 Ĭ
2. 21-25 Ĭ
3. 26-30 Ĭ
4. 31-35 Ĭ
5. 36-40 Ĭ
6. 41-45 Ĭ

SECTION 2: CONTRACEPTION (tick one)

a. CURRENT FAMILY PLANNING METHOD
    1. ORAL CONTRACEPTIVES Ĭ
    2. INJECTABLE Ĭ
    3. OTHERS Ĭ
b. FAMILY PLANNING METHODS IN THEPAST SIX (6) MONTHS
    1. NATURAL Ĭ
    2. MALE/FEMALE CONDOMS Ĭ
    3. SPERMICIDES Ĭ
    4. ORAL CONTRACEPTIVES Ĭ
    5. INJECTABLES Ĭ
    6. INTRAUTERINE DEVICES Ĭ
    7. OTHERS Ĭ
c. DURATION OF CONTRACEPTIVE USE (FOR THOSE ON DMPA)
    1. < 3 MONTHS Ĭ

2. 3-6 MONTHS Ĭ
3. 7-12 MONTHS Ĭ
4. >12 MONTHS Ĭ

SECTION 3: PHYSICAL EXAMINATION (tick one)

1. GENERAL EXAMINATION
   a. PALLOR PRESENT Ĭ ABSENT Ĭ
   b. JAUNDICE PRESENT Ĭ ABSENT Ĭ
   c. EDEMA PRESENT Ĭ ABSENT Ĭ
   d. FEVER PRESENT Ĭ ABSENT Ĭ
   e. LYMPHADENOPATHY PRESENT Ĭ ABSENT Ĭ
2. SYSTEMIC EXAMINATION
   a. CNS NORMAL Ĭ ABNORMAL Ĭ
   b. RESP SYSTEM NORMAL Ĭ ABNORMAL Ĭ
   c. CVS NORMAL Ĭ ABNORMAL Ĭ
   d. GIT NORMAL Ĭ ABNORMAL Ĭ
   e. UROGEN SYSTEM NORMAL Ĭ ABNORMAL Ĭ
   f. PELVIC EXAMINATION INSPECTION
      i. MONS PUBIS NORMAL Ĭ ABNORMAL Ĭ
      ii. VULVA NORMAL Ĭ ABNORMAL Ĭ
      iii. PERIANAL NORMAL Ĭ ABNORMAL Ĭ
      iv. VAGINA NORMAL Ĭ ABNORMAL Ĭ
      v. CERVIX NORMAL Ĭ ABNORMAL Ĭ
      vi. GENITAL ULCERS PRESENT Ĭ ABSENT Ĭ

SECTION 4: SPECIMENS COLLECTED

a. 4ML VENOUS BLOOD ON EDTA BOTTLE FOR LYMPHOCYTE COUNT DETERMINATION
   i. CD4 COUNT : _____
   ii. CD8 COUNT : _____
   iii. TOTAL LYMPHOCYTE COUNT _____

b. CERVICOVAGINAL LAVAGE FLUID(FOLLICULAR PHASE)
   i. VOLUME IN MLS _____

ii. MACROSCOPIC APPEARANCE (tick one)

1. CLEAR Ĭ
2. BLOOD STAINED Ĭ
3. OTHER Ĭ

SECTION 5: RESULTS (VIRAL LOADS CONCENTRATION (COPIES/ML)

1. NOT DETECTED (BELOW ASSAY THRESHOLD) _____
2. DETECTED (<40 COPIES/ML) _____
3. CONCENTRATIONS OF 40 ï 10MILLION _____
4. >10MILLION COPIES _____

APPENDIX II: INFORMED CONSENT INFORMATION

Used in the previous study on effects of depomedroxyprogesterone acetate on cervical immune activation (7).

COMPARATIVE STUDY OF PROINFLAMMATORY CYTOKINE PROFILE OF HIV POSITIVE WOMEN ON DEPOT MEDROXYPROGESTERONE ACETATE

FACES KENYA * UNIVERSITY OF NAIROBI

Please take as much time as you want to read this form, ask questions, and talk about this project with family or friends.

What is this project about?

This project aims to investigate immune system factors in the mucosa (lining) of the lower female genital tract to determine whether there are factors, such as inflammation which is a process by which the body reacts to pathogens and other potentially threatening process and results in healing and repair. We would like to investigate whether progesterone based contraceptives contributes to this process in HIV positive women.

Why are we doing this project?

Cytokines are protein compounds which are important for intercellular communications between cells of the immune system. They have various functions; those which mediate the inflammatory process are known as the proinflammatory cytokines. The proinflammatory cytokine levels may give us an understanding of the factors that may influence the risk of HIV spread in women using depomedroxyprogesterone acetate contraceptives. It also helps scientists to understand the between HIV positive and negative persons concerning genital tract immunology factors and the influence of contraceptives. This information is lacking particularly in Sub-Saharan Africa.

What are the benefits to the study participants and the community?

We will undertake diagnosis for infectious diseases of the genital tract and on making a diagnosis treatment will be provided. Cervical smears will be done and findings communicated to the study participants. Scientific information derived from this study will be useful in understanding immune factors of the genital tract, will be of benefit for the scientific community as a whole particularly in the field of reproductive health.

How will the samples be used?

Over the next two months the samples will undergo screening for infectious disease involving the genital tract. Proinflammatory cytokine profiles will then be established to determine mucosal immune factors which will give us an insight in the role of depomedroxyprogesterone acetate in HIV and AIDS. Samples will be retained for a period of 5 years for future studies in mucosal immunity.

What will happen if I decide to give a sample?

We will request you to abstain from sexual activity or intravaginal douching for at least 48 hours prior to sample collection. However, if you choose not to abstain then you may use a condom during sexual activity. We will ask you about your demographic information and menstrual history for the past six months in order to establish your menstrual cycles. We will use all this information to decide whether you can give a sample, but we will not keep any of this information or put it in the database. We will then draw about [4 ml] of blood from your arm. A gynaecologic exam will then be done and a cervical smear, swab and cervicovaginal lavage fluid will be collected. All of this will take about 1 hour. We will send your sample to the FACES laboratory for preliminary analysis and the Immunology Laboratory, School of Medicine, University of Nairobi for analysis. Samples collected will be stored for a period of 5 years to aid in validation of the test. The institution may send the samples to other researchers for use in future mucosal immunity studies as described in this form. The researchers will have to follow all U.S. and international laws and guidelines that apply to research. All studies using the cell lines will have to be approved by the institutional Ethical Review Board. An ERB is a committee similar to the one that approved this project to make sure that your rights were protected. Also, a Community Advisory Group will be set up for each community that takes part in this project. This group will include people from your community and will make sure that future studies using your communityôs samples are similar to ones described in this form. This group will also suggest ways to do those studies to limit any possible harm to your community.

Will there be any costs or payments?

It will not cost you anything to be a part of this project. We will give you KSh 200 for your time, travel, and inconvenience if you come in to give us a sample. The institution does not let anyone sell material from samples or cell lines. Also, because the cell lines will not have

names on them, neither the researchers nor anyone at the Repository would know if your sample was even used. So you will not get any additional payment if you take part in this project.

How will you protect my privacy?

We will protect your privacy in several ways. While FACES will keep your signed consent form, nobody else will see it. We will not keep your name with your sample or give your sample a code number that could identify you. So nobody at the Repository or who studies your sample will know that it came from you. Also, we will collect more samples than we will use. This way nobody, not even you or us, will know if your sample was used or if any information in the database came from you. (Samples that are not used will be disposed of in standard ways).

What are the benefits of giving a sample?

Your samples will be screened for infectious disease and when diagnosed, treatment will be offered in line with institutional guidelines. Researchers will study these samples for many years to learn about health and disease. This research will eventually benefit the health of people around the world.

What are the risks of giving a sample?

Drawing blood has very minor risks. These include brief pain, slight bruising, dizziness or fainting, and (very rarely) infection where the needle goes in. Lower genital tract sampling may be uncomfortable but has minor risks.

Are there any risks to my community or group?

Information on the demographic characteristics of participants will be included with the samples, in the database, and in the published report. In future studies, researchers may find that certain mucosal immunity variations appear more often in people from your group than in people from other groups, and that these variations are more common in people with a certain disease. This may make some people look down on your group unfairly. Some people may use the information from the cervicovaginal lavage cytokine profile study or from future studies using the published to exaggerate differences between groups for prejudiced or other bad reasons. Others may use the information to downplay differences between groups, to say that all peopleôs mucosal immune characteristics are about the same, so we donôt need to respect

the special concerns of different groups. Biology does not provide a reason for prejudice, but discrimination does exist. We will work to make sure that the ethnic or geographic identity of your community is described as carefully as possible--in the sample collection, in the database, in and in any articles researchers write about the trial.

Can I change my mind after I give a sample?

Giving a sample is completely up to you. You will not lose any benefits if you choose not to give a sample. However, after you give a sample you cannot take it back or take any information out of the database. However, in this case your sample and results will not be included in the analysis

How will I find out what happens with this project?

Because your sample will not have your name on it, we will not be able to give you individual results from this research. However, we will update your community through FACES Nyanza programme on how researchers are using the cervicovaginal lavage samples and your communityôs samples and what they are learning about health and disease. The results of the infection screen will be communicated to your attending physician for clinical care. The results will also be analyzed and published in a journal and on the FACES-Nyanza web site. A dissertation will also be written and will be kept in the University of Nairobi- School of Medicine library, and at the Department of Human Pathology library.

APPENDIX III: CONSENT FORM

Used in the previous study on effects of depomedroxyprogesterone on cervical immune activation (7).

Please read the paragraph below, think about your choice, and sign if you agree:

I agree to participate and undergo a medical examination and allow female genital tract sampling and blood for researchers to use for the proinflammatory cytokine profile study and in other approved studies of the type described in the form, and other studies in mucosal immunity. I have read or listened to the information, I have asked any questions I had, and all my questions were answered. I know that giving a sample is my choice.

Your Signature _____

Date _____

I agree to the storage of these samples for a period of not more than five (5) years, and I understand that any study conducted on these samples must undergo review and ethical approval from an ethical review committee.

Your Signature_____

Date_____

Copy given to participant: _____Yes

Who can I talk to if I have questions or problems?

If you have questions about this sample collection, contact:

(PI) DR WALONG EDWIN OLOO (phone) +254738590623, Email: edwin.owino@students.uonbi.ac.ke.

If you have questions about your rights as part of this research project, contact:

(ERC) KENYATTA NATIONAL HOSPITAL-UNIVERSITY OF NAIROBI ETHICAL REVIEW COMMITTEE.PO BOX 20723 KNH-NAIROBI. (Phone) (+254)20 726300. E-Mail:KHHplan@Ken.Healthnet.org

APPENDIX IV: TEST PRINCIPLES AND PROCEDURES

STANDARD OPERATING PROCEDURE

Version: 1.20
ABBOTT Real-time HIV-1 TEST for Viral Load

1. **PURPOSE**

This SOP defines the steps to be followed in processing human plasma samples for quantitative RNA PCR (Viral Load).

2. PRINCIPLE

2.1. The Abbott Real-t*i*me HIV-1 assay is an *in vitro* reverse transcription-polymerase chain reaction (RT-PCR) assay for the quantitation of Human Immunodeficiency Virus type 1 (HIV-1) in human plasma from HIV‑1 infected individuals. The Abbott Real-t*i*me HIV-1 assay is intended for use in conjunction with clinical presentation and other laboratory markers as an indicator of disease prognosis and for use as an aid in assessing viral response to antiretroviral treatment as measured by changes in plasma HIV-1 RNA levels. This assay is not intended to be used as a screening test for HIV-1 or as a diagnostic test to confirm the presence of HIV-1 infection.

2.2. The Abbott Real-t*i*me HIV-1 assay uses RT-PCR to generate amplified product from the RNA genome of HIV-1 in clinical specimens. An RNA sequence that is unrelated to the HIV-1 target sequence is introduced into each specimen at the beginning of sample preparation. This unrelated RNA sequence is simultaneously amplified by RT-PCR, and serves as an internal control (IC) to demonstrate that the process has proceeded correctly for each sample. The amount of HIV‑1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labeled oligonucleotide probes on the Abbott *m*2000*rt* instrument. The probes do not generate signal unless they are specifically bound to the amplified product. The amplification cycle at which fluorescent signal is detected by the Abbott *m*2000*rt* is proportional to

the log of the HIV-1 RNA concentration present in the original sample.

3. REFERENCES

3.1. Abbott Molecular Operation Manual for M2000rt

4. RESPONSIBILITY

4.1. It is the responsibility of the Laboratory Director/ Manager to ensure that this SOP is followed or adhered to by the Laboratory technologists

4.2. The Laboratory technologist is responsible for processing and handling of the specimen until results are generated.

5. DEFINATION

5.1. CD4

6. SAFETY PRECAUTIONS

6.1. Use universal precautions with all specimens. Adhere to the following safety practices:

6.2. Wear laboratory coats and gloves when processing and analyzing specimens.

6.3. Never pipette by mouth. Use safety-pipetting devices.

6.4. Handle and manipulate specimens (e.g., aliquot, add reagents, vortex, and aspirate) in a Class I or II biological safety cabinet. Centrifuge specimens in safety carriers.

6.5. After working with specimens remove gloves and wash hands with soap and water.

7. MATERIALS AND EQUIPMENTS

7.1. Abbott Real-t*i*me HIV-1 Amplification Reagent Kit

7.2. Abbott Real-t*i*me HIV-1 Control Kit

7.3. Abbott Real-t*i*me HIV-1 Calibrator Kit

7.4. Micro Amp Reaction tubes and caps

7.5. Tray/Retainers and Base

7.6. Multi-channel pipette

7.7. Pipettor (200ul capacity)

7.8. Disposable powder free gloves

7.9. Plastic reseal able bags

7.10. 2.0ml sterile polypropylene, non-siliconized, conical screw-cap tubes

7.11. 2.0ml and 5ml Tube racks

7.12. Sterile fine-tips RNase free

7.13. Sterile disposable serological pipettes (5ml, 10ml and 25ml)

7.14. Magnetic stands

7.15. Pipettor (capacity 10-1000ul).

7.16. Aerosol barrier RNase-free tips

7.17. Vortex mixer

7.18. Powder free gloves

7.19. 5ml tube capacity heat block

7.20. 2ml tube capacity heat block

8. PROCEDURE

8.1. **Reagent Preparation**: Performed in; Pre-Amplification ï RNA Extraction area.

8.1.1. Thaw Internal Control, Samples, Standards and Kit controls to room temperature.

8.1.2. Vortex briefly Internal Control, Samples, Standards and Kit controls

8.1.3. Prepare Working Lysis Reagent. Vortex **HIV-1 IC** for 5-10 seconds before use. For each batch of 24 specimens and controls, add 600ul of HIV-1 IC to one bottle of **HIV-1 LYSIS** and mix well.

8.2. **Specimen and Controls processing**

8.2.1. Determine and label the appropriate number of reaction tubes needed for patient specimen, Standards and control testing. Place the tubes in the Micro Amp tray and lock in place with retainer.

8.2.2. Add 100ul Iron particles into each 5ml reaction tube using a repeat pipette with aerosol barrier tips.

8.2.3. Add 2.5ml Prepared Lysis Reagent into each 5ml reaction tube using a repeat pipette with aerosol barrier tips

8.2.4. Add 200 µl to 1ml sample into each 5ml reaction tube using an aerosol barrier tips and mix 3-5 times to completely homogenize the lysis reagent and the

sample.

NOTE**: **Individual samples are added to respective individual tube with volume dependent on the protocol volume selected.

8.2.5. Incubate the mixture at 50±2ºC on the 5ml heat block and time for 20minutes.

8.2.6. Remove the tubes and place them in the magnetic capture stand for 2minutes.

8.2.7. Carefully remove the lysate from each tube using sterile disposable Pasteur pipettes, remove the fluid as much as possible. Do not disturb the captured magnetic particles. Open one tube at a time.

8.3. **First Wash**

8.3.1. Transfer 12X75mm tubes to a non-magnetic rack immediately after removal of lysis buffer, add 700ul of *m*wash 1 to each tube and re-suspend the magnetic particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.3.2. Transfer wash fluid and particles to a labeled 1.5ml screw top tube.

8.3.3. Place 1.5ml tubes in a magnetic capture stand for 1 minute.

8.3.4. Carefully remove *m*wash 1 from each tube using extended aerosol barrier pipette tips, remove the fluid as much as possible, do not disturb the captured magnetic particles, open one tube at a time.

8.3.5. Transfer 1.5mm tubes to a non-magnetic rack immediately after removal of lysis buffer, add 700ul of *m*wash 1 to each tube and re-suspend the magnetic particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.3.6. Transfer wash fluid and particles to a labeled 1.5ml screw top tube.

8.3.7. Place 1.5ml tubes in a magnetic capture stand for 1 minute.

8.3.8. Carefully remove *m*wash 1 from each tube using extended aerosol barrier pipette tips, remove the fluid as much as possible, do not disturb the captured magnetic particles, open one tube at a time

8.4. **First Wash 2**

8.4.1. Transfer 1.5mm tubes to a non-magnetic rack immediately after removal of mwash 1 buffer, add 700ul of *m*wash 2 to each tube and re-suspend the magnetic

particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.4.2. Transfer wash fluid and particles to a labeled 1.5ml screw top tube.

8.4.3. Place 1.5ml tubes in a magnetic capture stand for 1 minute.

8.4.4. Carefully remove *m*wash 2 from each tube using extended aerosol barrier pipette tips, remove the fluid as much as possible, do not disturb the captured magnetic particles, open one tube at a time.

8.5. **Second Wash 2**

8.5.1. Transfer 1.5mm tubes to a non-magnetic rack immediately after removal of mwash 2 buffer, add 700ul of *m*wash 2 to each tube and re-suspend the magnetic particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.5.2. Transfer wash fluid and particles to a labeled 1.5ml screw top tube.

8.5.3. Place 1.5ml tubes in a magnetic capture stand for 1 minute.

8.5.4. Carefully remove *m*wash 2 from each tube using extended aerosol barrier pipette tips, remove the fluid as much as possible, do not disturb the captured magnetic particles, open one tube at a time.

8.6. **Elution**

8.6.1. Transfer 1.5mm tubes to a non-magnetic rack immediately after removal of mwash 2, add 25ul of *elution buffer* to each tube and re-suspend the magnetic particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.6.2. Place lysis tubes in the 75ºC heating block, incubate for 20minutes.

8.6.3. Transfer 1.5mm tubes to a non-magnetic rack, add 63ul of *m*wash 2 to each tube and re-suspend the magnetic particles by aspiration, wash the particles from the side of the tube, if necessary. Avoid foaming.

8.6.4. Place 1.5mm tubes to a magnetic capture stand for 1 minute

8.6.5. Remove the elute and transfer to a fresh RNAse free 1.5ml tube or 96 well polypropylene tray (PCR tubes)

8.7. **Master Mix Set Up**

8.7.1. Thaw assay Reagent Pack

8.7.2. Mix reagents by inverting 5 to 10 times

8.7.3. Prepare the MMX by adding 270ul of the HIV-1 activation Reagent (Reagent 1) in the thermostable rTth DNA polymerase enzyme bottle (Reagent 3). Add 950ul of the HIV-1 Oligonucleotide reagent (reagent 2) in the thermostable rTth DNA polymerase enzyme bottle (Reagent 3).

8.7.4. Prepare 96-well Optical reaction plate; place an Abbott 96-well Optical reaction plate in a StrataCooler 96.

8.7.5. Dispense amplification master mix, dispense 50ul aliquots 0f the amplification master mix into the 96-well plate

8.7.6. Add 50ul of sample elute to the 96-well plate, mix by pipetting up and down 3-5 times. Uses a separate pipette tip for each sample elute transfer.

8.7.7. Seal the 96-well plate using an Abbott m2000rt Optical Adhesive Cover and fix it with Optical Adhesive Cover Applicator. Do not touch the surfaces of the cover.

8.7.8. Separate the strips at the edges along the perforated line and transfer the plate to the amplification machine.

9. REVERSE TRANSCRIPTION, AMPLIFICATION AND DETECTION

(Performed in: PCR Amplification and Detection Area)

***NOTE**: Turn on the m2000rt thermal cycler 15 minutes prior to beginning the amplification for initialization.*

9.1. Place the tray/retainer assembly into the thermal cycler block.

9.2. Program the m2000rt thermal cyclerby creating a method as follows:

Stage 1; RT:

Step 1 (1 cycles) 30 min 59^0C

Stage 2; low stringency PCR

Step 1; Temperatures; 95^0C

Time; 40seconds

Step 2; Temperatures; 46^0C

Time; 30seconds

Cycles 4

Stage 3; High stringency PCR

Step 1; Temperatures; 92^0C

Time; 30seconds

Step 2; Temperatures; 60^0C

Time; 30seconds

Cycles 6

Stage 4; PCR and Detection

Step 1; Temperatures; 92^0C

Time; 30seconds

Step 2; Temperatures; 56^0C

Time;20seconds+ 2sec Auto Increment

Step 3; Temperatures; 35^0C

Time; 40seconds

Cycles 37

9.3. Start the Method program. The program runs for approximately Three hours.

10. RESULTS

10.1. The m2000rt will generate a standard curve from the six calibrators run (3 Cal A and 3 Cal B) and the log numbers of individual patients will be calculated against the standard curve automatically.

10.2. The results are then generated as follows;

10.2.1. Not Detected (Below assay threshold)

10.2.2. Detected, <40 Copies/ml (600ul-1ml protocol)

10.2.3. Concentration of 40 to 10,000,000M Copies/ml

10.2.4. >10M Copies