EFFECTS OF HUMAN HERPESVIRUS 7 INFECTION ON EXPRESSION LEVELS OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 RECEPTORS ON CD4+ T CELLS AMONG HIV-1 HIGHLY EXPOSED SERO-NEGATIVE PERSONS IN NAIROBI KENYA

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF THE DEGREE IN MASTER OF SCIENCE IN BIOTECHNOLOGY

BY

AKISO MATRONA MBENDO

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DECLARATION

I, Akiso Matrona Mbendo, hereby declare that this thesis is my original work and has not been presented for a degree in any other university. Relevant literature has been duly referenced.

Signature…………………………….. **Date**………………………………….

Name: Akiso Matrona Mbendo

Reg: I56/82443/2012

SUPERVISORS

DR. JULIUS OYUGI,

MSc, PhD, Senior Lecturer,

Department of Medical Microbiology,

University of Nairobi

Signature……………………………… **Date**………………………………

PROF. JAMES O. OCHANDA,

Center for Biotechnology and Bioinformatics,

University of Nairobi

Signature………………………………. **Date**……………………………….

LIST OF ABBREVIATIONS

ABI	Applied Biosystems
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
CCR	Chemokine Co-Receptor
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
CVL	Cervical Vaginal Lavage
CXCR	Chemokine (C-X-C motif) Receptor
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr Virus
EDTA	Ethylene-Diamine-Tetraacetic Acid
ERC	Ethics and Research Committee
FACS	Fluorescence activated cell sorting
FCS	Flow Cytometry Data File Format Standard
FITC	Fluorescein isothiocyanate
HCMV	Human Cytomegalovirus
HESN	Highly Exposed Seronegative
HHV	Human Herpesvirus
HIV	Human Immunodeficiency Virus
ILRI	International Livestock Research Institute
KNH	Kenyatta National Hospital
MFI	Median Fluorescent Intensity
mIgG	Mouse immunoglobulin
NNRTI	Non-Nucleoside Reverse Transcriptase Inhibitors
NRTI	Nucleoside Reverse Transcriptase Inhibitors
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PECy5	Phycoerythrin cyanine 5
PI	Protease Inhibitors

RT PCR	Real Time Polymerase Chain Reaction
TBE	Tris Borate EDTA
UNAIDS	United Nations AIDS
UNITID	University of Nairobi Institute of Tropical and Infectious Diseases
UoN	University of Nairobi
WHO	World Health Organization

LIST OF ACRONYMS AND SYMBOLS

$<$	less than
$\geq$	equal or greater than
µg	microgram
µl	micro liter
µM	micro molar
g	gram
min	minute
ml	milliliter
mM	mill molar
nM	nano molar
p	Symbol for level of significance
r	Pearson's Coefficient Correlation symbol for correlation strength
sec	second
vs	versus

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ABSTRACT

Background: Remaining as one of the infectious agents associated with major global health burdens, to date, there is no effective vaccine/drug to HIV and the available anti-retroviral therapy (ART) only prolongs the lives of the infected people. Exposure to HIV has been shown to either lead to established infection or no infection. Why some people don't get infected has not been fully understood. Studies on other viruses that modulate HIV infection may shed some light on this unique phenomenon. Human herpesvirus 7 (HHV7) is the only other virus known to use the CD4 surface glycoprotein exclusively as its primary receptors into the CD4$^+$ T cells. This virus has been shown to down-regulate surface expression of these receptors in *in-vitro* studies hence interfere with HIV infection into these cells.

Objectives: The study aimed at assessing the impact of HHV7 infection on expression of CD4$^+$ T cell surface receptors used by HIV for entry into the host cells.

Methodology: This was a cross sectional cohort study where HIV discordant couples aged between 18 and 50 years in the Pumwani discordant couples' cohort were recruited. A calculated sample size of 125 study participants was used. Participants were categorized into Highly exposed seronegative (HESN), HIV positive and HIV negative controls. Laboratory procedures included PCR and Flow cytometry to detect and quantify HHV7 genome and the surface expression levels of the CD4$^+$ T cell surface receptors in the genital mucosa respectively.

Results: HHV7 was more prevalent in males and the viral loads were significantly higher in the males regardless of their HIV status. CD4+ T cell frequencies were higher in the HHV7+ controls. This was also observed in cases that were negative for both HHV7 and HIV. There was a strong positive correlation between HHV7 viral loads and CD4+ T cell frequencies. The expression levels of the CD4 receptors were reduced in HHV7+ subjects. A negative correlation was observed between HHV7 viral loads and CD4 receptor expression. CCR5 expression was higher in both HHV7+ and HIV+ subjects. However, a negative correlation was observed between HHV7 viral loads and CCR5 expression levels.

Conclusion: The study results support findings from previous in-vitro studies which demonstrated HHV7 induced inhibition of HIV-1 infection into the CD4+ T cells. In addition, an increase in the CD4+ T cell frequencies was observed in the HHV7+ control population. In conclusion, the study proposes that HHV7 could be the agent behind the HIV infection resistance of the HESN group in the study population.

1.0　INTRODUCTION

Human immunodeficiency virus (HIV) remains one of the infectious agents associated with major global health burdens. It has emerged from being a small health problem in the 1980s to being among the leading causes of mortality to date (Ortblad, Lozano, and Murray 2013). Since the time it was discovered in 1984, deaths from HIV/Acquired immunodeficiency syndrome (AIDS) have increased from 0.30 million in 1990 to 1.50 million in 2010 having its peak in 2006 of 1.7 million deaths (Lozano et al. 2012). In 2010, HIV/AIDS was among the leading causes of death (Lozano et al. 2012). As per the Joint United Nations Programme on HIV and AIDS (UNAIDS) 2013 report, a total of 35.3 million people were living with HIV/AIDS while there were 2.3 million new infection cases and 1.6 million deaths resulting from Acquired Immunodeficiency Syndrome (AIDS) in 2012 (UNAIDS. 2013). This report shows a global decline in the number of HIV/AIDS deaths by 30% since 2005, a global decline in new infections by 33% since 2001 and a decline by 50% of new infections in 26 low and middle income countries (Henry J. 2013). Further, the world health organization (WHO) HIV/AIDS fact sheet number 360 shows a global decline in the total number of people living with HIV (from 35.3 million people at the end of the year 2012 to 35 million people at the end of the year 2013). Similarly, the same report shows a global decline in the total number of deaths and new infections (from 1.6 to 1.5 million deaths and from 2.3 to 2.1 million new infection cases) between the ends of the years 2012 and 2013. This may be due to the easy access to the antiretroviral therapy (ART) and awareness campaigns on HIV/AIDS.

Africa carries 11% of the world's population, however, approximately 67% of the people living with HIV/AIDS globally are in Africa (DevelopAfrica 2014) with sub-Saharan Africa accounting for almost 70% of the global new HIV infection cases ("WHO | HIV/AIDS" 2015a). This unfortunate fact has led to Africa being named the epicenter of the HIV pandemic.

Substantial global actions have emerged with regards to this pandemic with new institutions having been formed in search for HIV/AIDS cure and prevention programmes put in place. Scientific efforts have led to the development of the life prolonging antiretrovirals (ARVs). All these efforts have contributed to the decline in the number HIV/AIDS related deaths and new infections. However, HIV/AIDS still remains among the top killer disease. In addition, the HIV epidemic not only affects the health of individuals but also impacts households, communities, and the development and economic growth of nations (AIDS.gov 2014). The absence of a

curative drug for HIV/AIDS therefore calls for more efforts in scientific research. Studying other pathogens that interfere with HIV infection may aid in providing information that can lead to either improving treatment or improving vaccine development.

Human Herpesvirus 7 (HHV7) is the only other virus known to use the CD4 surface glycoprotein as its primary receptor into the $CD4^+$ T cells and induces an abrupt down regulation of surface expression of these receptors (Paola Secchiero et al. 1998). This has been shown to interfere with HIV infection into these cells (Lisco et al. 2007). This is because HIV also uses the CD4 surface glycoprotein as the primary receptor on the $CD4^+$ T cells. Several studies have shown that there are individuals who despite continued exposure to HIV have remained resistant to its infection. Determining the reason behind this may give a hopeful clue to developing an effective drug to HIV. This study aimed at assessing whether infection with HHV7 contributes to the resistance to infection by HIV-1 among HIV discordant couples.

2.0   LITERATURE REVIEW

2.1   HUMAN HERPES VIRUS 7 (HHV7)

HHV7 was first isolated in 1990 from activated $CD4^+$ T lymphocytes purified from peripheral blood mononuclear cells of healthy donors (Frenkel et al. 1990). It belongs to the beta-herpesvirus subfamily with its primary infection occurring early in life (Kempf 2002). The virus is closely related to HHV6 and human cytomegalovirus (HCMV) but distinct from the Epstein barr virus (EBV) (Ablashi et al. 1995). Infection with HHV7 can sometimes cause exanthema sabitum (a disease in children mostly under 2 years old manifested by transient rash) and has also been associated with a number of other diseases such as encephalopathy (Tanaka et al. 1994). HHV7 is ubiquitous with a nearly universal prevalence in persons older than 6 years (Wolz, Sciallis, and Pittelkow 2012).

2.2   HHV7 INFECTION

Upon infection, HHV7 may establish a latent infection in the host cell (Caserta et al. 2007) or a persistent infection which last for the life time of the host (Chapenko et al. 2000). The virus is thought to be reactivated in immunosuppressed conditions due to diseases or therapy and in conditions leading to T cell activation (Chapenko et al. 2000; Frenkel et al. 1990). The primary root of entry into the human host of this virus is yet to be determined but the salivary system is the only source currently perceived to produce infectious HHV7 due to the high frequency at which the infectious virus is shed from the saliva (Minarovits, Gonczol, and Valyi-Nagy 2006).

It has been shown that the cellular receptor for HHV7 is the CD4 antigen expressed at high levels on the surface of a subset of mature T lymphocytes ($CD4^+$ cells) (P Secchiero et al. 2001). Upon infection in to the target cell, it is assumed that the virus down regulates CD4 antigen expression on the target cell surface (Furukawa et al. 1994). In another study by Secchiero and his colleagues in 1998, they also showed that HHV-7 interacts with the CXC chemokine receptor 4 (CXCR4) in a unique manner and causes a rapid and persistent down regulation of its surface expression (Paola Secchiero et al. 1998). CXCR4 is a chemokine receptor expressed on the surface of all hematopoietic cells including $CD4^+$ T lymphocytes. Due to this circumstance, HHV7 infection has been perceived as a hindrance to Human immunodeficiency virus type 1 (HIV-1) infection as HIV-1 also uses the CD4 antigens as the primary receptors for entry into the target cells (Zhang et al. 2000).

In an in vitro study by Lisco et al in 2007, HHV7 induced inhibition of HIV-1 was sufficient and could be compared to inhibition by cyclotriazadisulfonamide which is a synthetic macro cycle that specifically modulates expression of CD4 (Lisco et al. 2007).

2.3   HUMAN IMMUNODEFICIENCY VIRUS (HIV)

HIV is a lentivirus (slowly replicating virus) that infects cells of the immune system (CD4$^+$T cells, macrophages and dendritic cells). The virus then leads to the reduction in number of the CD4$^+$ cells in a number of ways such as, apoptosis of uninfected bystander cells, lysis of the infected cells through the viral lytic cycle and recognition and killing of the infected cells by the cytotoxic T lymphocytes (CTLs)(Wikipedia. 2013). Reduction of the CD4$^+$ T cells below critical levels leads to loss of cell mediated immunity hence rendering the body susceptible to opportunistic diseases. This condition is termed acquired immunodeficiency syndrome (AIDS).

Since the beginning of the epidemic, approximately 70 million people have have been infected with HIV and about 35 million of these have died from HIV/AIDS (WHO. 2013b). By the end of 2011, 34 million people were living with the virus globally and currently there is an estimation of 0.8% adults aged between 15-49 years living with the virus. However, the burden of the epidemic varies considerably between countries and regions (WHO. 2013b). In Kenya, despite the decrease in number from 2001, there were approximately 1.5 million people living with HIV/AID in 2012 and Kenya was ranked fourth after South Africa, Nigeria and India (Index Mundi 2014).

To date, there is no cure for HIV/AIDS. However, scientific progress has guided to the development of life prolonging antiretroviral therapy (ART). This involves the usage of a combination of drugs of which each blocks the virus at different stages of its development. These drugs include the Non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), nucleoside reverse transcriptase inhibitors (NRTIs), entry or fusion inhibitors and Integrase inhibitors. NNRTIs and protease inhibitors work by disabling the enzymes reverse transcriptase and protease respectively which are needed by HIV to make copies of it. NRTIs are faulty versions of building blocks that HIV needs to make copies of it while entry inhibitors block HIV's entry into CD4 cells. Integrase inhibitors work by disabling integrase, a protein that HIV uses to insert its genetic material into the genetic material of CD4 cells. Additionally, scientific progress has led to the understanding of virus-host cellular interactions.

3.0 HYPOTHESIS, RESEARCH QUESTION, OBJECTIVES AND SIGNIFICANCE

3.1 HYPOTHESIS

Infection of CD4+ T cells with HHV7 leads to down regulation of the CD4 surface receptors hence contributes to resistance to HIV-1 infection.

3.2 RESEARCH QUESTION

Is the infection with HHV7 a contributor to resistance to HIV-1 infection?

3.3 OBJECTIVES

3.3.1 General objective

This study aimed at assessing the impact of HHV7 infection on expression levels of CD4+ T cell surface receptors used by HIV-1 to gain entry into the cells among discordant couples attending the Pumwani maternity clinic in Nairobi Kenya.

3.3.2 Specific objectives

The study specifically:

1) Assessed the differences between HHV7 viral loads in the genital mucosa of HIV-1 resistant persons and HIV-1 non resistant persons.
2) Determined the correlation between HHV7 viral loads and expression levels of CD4+ T cell surface receptors (CD4 and CCR5) in the genital mucosa of the study subjects.
3) Determined the association between HHV7 infection state and expression levels of $CD4^+$ T cell surface receptors (CD4 and CCR5) in the genital mucosa of the study subjects

3.4 SIGNIFICANCE OF THE STUDY

As mentioned earlier, HIV/AIDS accounts for a great number of deaths. Great scientific efforts have led to the development of life prolonging drugs. Despite all these efforts, paucity of knowledge on the HIV virus has hindered the development of effective HIV drugs/vaccines to completely cure the infected people (Marmor et al. 2006). This has therefore led to scientists looking for alternative approaches in this fight. Studying other viruses that use similar host cells and cellular receptors as HIV may lead to the success of this search.

It has been observed through a number of studies that there are individuals who despite continued exposure to HIV have remained resistant to its infection. From studies carried in Africa and Thailand, this situation was exhibited in a small population of individuals, especially among the commercial sex workers (Marmor et al. 2006). More studies into why these persons remain resistant to the virus infection may give a hopeful clue to developing an effective HIV drug/vaccine (Pancino et al. 2010). Already a number of genetic variations among individuals have been shown to play a role in this resistance. This includes mutations in the chemokine co-receptor-5 (CCR5) which is used by some strains of HIV-1 (Macrophage tropic (M-tropic) strains) in combination with CD4 to gain entry into the host cell. HIV specific T and B cell responses have also been detected in some HIV-1 seronegative persons (Marmor et al. 2006). This indicates that these individuals have been exposed to the virus but somehow have cleared it from their bodies.

HHV7 is the only other known virus that uses the CD4 surface glycoproteins exclusively as the main receptors on the CD4+ T cells. This study aimed at assessing whether infection with HHV7 contributes to the HIV-1 infection resistance in a cohort of highly exposed seronegative (HESN) discordant couples. From our results, we speculate that HHV7 may guide in the search for better HIV interventions.

4.0   METHODOLOGY

4.1   STUDY DESIGN

This was a cross-sectional study. The study recruited HIV discordant couples and HIV negative individuals who were not discordant couples. The later served as the control group. The discordant couples were drawn from the Pumwani Maternity Clinic discordant couples' cohort while the control group was drawn from the Pumwani community.

4.2   STUDY POPULATION AND RECRUITMENT

The study subjects were derived from a cohort of discordant couples in Pumwani area and visiting the Pumwani maternity clinic in Nairobi. This cohort was established in 1985. More than 3000 women had been recruited as of January 2011. About 10% of these couples are classified as HESN people. The study participants were fully informed on the study as per the informed consent form by the designated nurse. Upon full understanding of the study and all their questions answered to their satisfaction, the willing participants signed or marked 2 copies of the Informed Consent Form. This was to confirm that they had been informed about the study and voluntarily agreed to take part. One copy was theirs to keep and the other was kept in our confidential file. Those who did not wish to keep their copy signed or marked a form that stated they did not want to take it, and we kept it for them. The participants were then asked questions about their general health and medical examination were performed by the designated trained nurse at the clinic.

4.3   STUDY SITE

This study was carried out in two sites. The study subjects were recruited from the Pumwani area in Nairobi. Sample collection was done at the Pumwani maternity clinic by a trained nurse and a physician. Sample processing and all other laboratory procedures for this project were done at the University of Nairobi, Institute of Tropical and Infectious Diseases (UNITID) laboratories.

4.4   INCLUSION CRITERIA

The study recruited adults aged between 18 to 50 years. Upon being fully informed on the study, the willing subjects signed or marked an informed consent form to assure their willingness to take part in the study.

4.5   EXCLUSION CRITERIA

Subjects unable/unwilling to provide informed consent were not included in the study. Pregnant women confirmed by the urine test and subjects with AIDS related infections such as those with tuberculosis were also not recruited in the study.

4.6   SAMPLING AND SAMPLE SIZE CALCULATIONS

The sample size was calculated based on a previous prevalence research on pregnant women who secret HHV7 in the cervical secretions which was 10% (P = 0.1). The standard normal deviation was estimated at a 95% level of significance (Z_α = 0.05) while the margined of error (d) was estimated at 4% (d = 0.04). The sample size (N) was therefore calculated as follows:

N=(Z_α^2(1-P)P)/d^2

This formula gave a total of **216** persons. However, due to financial constraints, **125** subjects were recruited. Of these, 100 were discordant couples (DC) and 25 were HIV-1 negative people (the control group, not DC and either married or not). The 125 study subjects were categorized in six groups as in table 1.

Table 1: Study Subjects Categories

	Males	Females
HIV Positive DC	25	25
HIV Negative DC	25	25
Control Group	10	15

DC: Discordant Couples

4.7   ETHICAL CONSIDERATIONS

Ethical approval was sought from the Kenyatta National Hospital/ University of Nairobi Ethics and Research Committee (KNH/UoN ERC). The subjects were fully informed on the study with regards to the purpose of the study, risks, confidentiality, benefits and free will to participate as was indicated in the informed consent form. All willing subjects were then asked to voluntarily fill and sign or mark two copies of the consent forms with detailed information of the study. Questionnaires were then administered to collect demographic data.

4.8   SAMPLE COLLECTION, PROCESSING AND STORAGE

The samples collected were whole blood, cervical vaginal lavage (CVL), cervical mononuclear cells (CMCs), urine and semen. Whole blood was obtained from each subject using 10ml Ethylene-Diamine-Tetraacetic Acid (EDTA) vacutainer tubes after which peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation using lymphoprep and stored in liquid nitrogen. Plasma from these samples was stored at -80^0C. CVL and CMCs were obtained from each of the female subject while semen samples were obtained from each male subject. 10mls of urine samples were also obtained from all study subjects. CMCs were used for flow cytometry analysis to determine the expression levels of CD4+ T cell surface receptors. Semen and CVL samples were used for polymerase chain reaction (PCR) to detect and quantify the HHV7 genome.

4.8.1   Whole blood processing

Whole blood was processed to isolate PBMCs and plasma which were stored in liquid nitrogen and -80^0C freezer respectively. Briefly, the collected whole blood was centrifuged at 1600 rpm for 10 min and the plasma aliquoted into cryovials for storage at -80^0C. The remaining blood was then diluted by phosphate buffered saline (PBS) containing 2% fetal calf serum (FCS) (2% PBS). PBMCs were then isolated using lymphoprep in a clean 50ml conical centrifuge tube and stored in liquid nitrogen.

4.8.2   Processing of Cervical Vaginal Lavage (CVL)

CVL was obtained from female subjects by irrigating the cervix with 2ml of a non bacteriostatic sterile $1\times$ PBS and then aspirated into a sterile 15ml conical centrifuge tube. The CVL was centrifuged at 1000 rpm for 10 min and 1ml aliquots of the supernatant put into cryovials for storage at -80^0C. The pellet was also put into a cryovial and stored at -80^0C. The supernatant was used to detect and quantify cell free HHV7 genome by use of RT PCR method while the pellet was used to detect cell associated HHV7 genome using convectional PCR and gel electrophoresis.

4.8.3　Processing of Cervical Mononuclear Cells (CMCs)

CMCs were obtained from the female subjects using a sterile cytobrush and plastic scraper. Briefly, a cytobrush was inserted 1cm into the cervix and the collected cells placed into a sterile 50ml conical tube containing 10ml of 1× PBS. The plastic scraper was then used to scoop more cells from the cervix and placed together with the cytobrush. The cells were filtered and washed to remove most of debris and stained with fluorochrome conjugated monoclonal antibodies for flow cytometry.

4.8.4　Semen processing

Semen was obtained from the male subjects. The subjects were given condoms to use for a sexual intercourse with their partners after which they brought the samples to the clinic the following morning. Semen samples were centrifuged and the supernatants and the pellets stored as explained for the CVL.

4.8.5　Urine

Urine samples were at the clinic where the study subjects were given urine collection container cups with screw-on lids to collect the samples. The samples were then transported to the UNITID laboratories in a cool box and stored at -80^0 C.

4.9　LABORATORY TESTS

The collected samples were tested using three laboratory procedures so as to achieve the study objectives: The assays included RT-PCR for detection and quantification of cell free HHV7 genome, conventional PCR and gel electrophoresis for detection of cell associated HHV7 genome and Flow cytometry to determine expression levels of CD4+ T cell surface receptors.

4.9.1　DNA Extraction

DNA was extracted from the CVL and semen samples using QIAmp DNA Blood Mini Kit (Qiagen). Briefly, the samples were first equilibrated to room temperature (21^0C) before adding 200µl to a micro centrifuge tube containing 20µl of QIAGEN protease. To this 200µl of buffer AL was added and pulse-vortexing for 15 seconds. This mixture was then incubated at 56^0C for 10 minutes then centrifuged briefly to remove drops from the inside of the lid. 200µl of 96%

ethanol was then added to the sample and mixed by pulse-vortexing for 15 seconds. This mixture was then carefully applied to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 8000 rpm for 1 minute. The Mini spin column was then placed in clean 2 ml collection tube and the tube containing the filtrate discarded. To the QIAamp Mini spin column, 500µl of buffer AW1 was added and centrifuged again at 8000 rpm for 1 minute before placing the Mini spin column again in a clean 2 ml collection tube and discarding the tube containing the filtrates. 500µl of buffer AW2 was then added to the sample and centrifuged at 14000 rpm for 3 minutes and the spin column placed in a clean 1.5 ml micro centrifuge tube. 200µl of buffer AE was then added to the sample. This was incubated at room temperature (21^0C) for 5 minute before centrifugation at 8000 rpm for 1 minute. The eluted DNA in the 1.5 ml micro centrifuge tube was stored at -20^0C and the spin column discarded.

4.9.2 RT PCR

TaqMan RT PCR method was employed and the Denovo Biotechnology's HHV7 qPCR kit was utilized for the assay together with the Qiagen HotStarTaq Plus DNA Polymerase master mix kit. A negative control (water) and a positive control (HHV7 DNA) were included in the PCR runs. A 20µl reaction mix was prepared as in the table 2 below:

Table 2: Preparation of the Reaction Mix

Reagent	Volume (µl)	Final Concentration
10× PCR Buffer	2	1×
5× Q Solution	4	1×
10mM of each dNTP mix	0.4	200µM of each dNTP
25mM $MgCl_2$	0.4	2mM
20× Primer/Probe mix	1	1×
HotStarTaq Plus DNA Polymerase	0.1	2.5 units/reaction
Nuclease free water	7.1	
Template	5	
Final volume	**20**	

A standard curve was prepared from which the HHV7 genome from the test samples was quantified. The 10^{12} copies per ml of the HHV7 standard was serially diluted as illustrated in table 3 below and a standard curve was constructed using the 10^2 to 10^8 copies per ml range.

Table 3: HHV7 Standard Curve Dilution

Tube No.	Add	Into	Final copy number
1	1µl of the 10^{12} per ml standard	Tube 1 containing 9µl of nuclease free water	10^{11} per ml
2	1µl of tube 1 content	Tube 2 containing 9µl of nuclease free water	10^{10} per ml
3	1µl of tube 2 content	Tube 3 containing 9µl of nuclease free water	10^9 per ml
4	1µl of tube 3 content	Tube 4 containing 9ul of nuclease free water	10^8 per ml
5	1µl of tube 4 content	Tube 5 containing 9µl of nuclease free water	10^7 per ml
6	1µl of tube 5 content	Tube 6 containing 9µl of nuclease free water	10^6 per ml
7	1µl of tube 6 content	Tube 7 containing 9µl of nuclease free water	10^5 per ml
8	1µl of tube 7 content	Tube 8 containing 9µl of nuclease free water	10^4 per ml
9	1µl of tube 8 content	Tube 9 containing 9µl of nuclease free water	10^3 per ml
10	1µl of tube 9 content	Tube 10 containing 9µl of nuclease free water	10^2 per ml

The amplification was done with initial enzyme activation at 50^0C for 2 min and at 95^0C for 10 min. This was followed by 40 cycles of denaturation at 95^0C for 15 seconds and data collection at 60^0C for 60 seconds. The viral load was expressed as the copy number of viral genome per ml of the DNA extract.

4.9.3 Conventional PCR

Conventional PCR utilized the BioLab's One Taq 2x Master Mix with Standard Buffer protocol. Primers (HHV7 forward 5'-AGCGGTACCTGTAAAATCATCCA-3' and HHV7 reverse 5'-AACAGAAACGCCACCTCGAT-3') specifically targeting the AL strain of HHV7 (Lisco et al. 2007) were ordered from the Internal Livestock Research Institute (ILRI). A 20µl reaction mix was prepared in a 200µl PCR tube as in table 4 and the amplification protocol was as in table 5:

Table 4: Preparation of Conventional PCR Master Mix

Reagent	Volume (µl)	Final Concentration
One Taq 2× Master Mix with standard buffer	10	1×
HHV7 forward primer (3pmol/µl)	2	300nM
HHV7 reverse primer (3pmol/µl)	2	300nM
Nuclease free water	1	-
Template	5	-
Final volume	**20**	-

Table 5: Convention PCR Amplification Protocol

Number of cycles	Step	Time	Temperature (^{0}C)
1	Initial denaturation	1 min	94
40	Denaturation	30 sec	94
	Annealing	30 sec	51
	Extension	1 min	68
1	Final extension	5 min	68
1	Hold	10 min	4

4.9.4 Gel electrophoresis

A 1% agarose gel was made by dissolving 1g of agarose in 100ml of 1× Tris Boric EDTA (TBE) buffer: 10× TBE comprised 109g Tris base, 55g Boric acid and 4.65g EDTA in 1000ml double distilled water. 20µl of ethidium bromide was added to the gel. The gel was run for 45 min at a voltage of 120. Detection of amplicons was done using an ultra violet transilluminator.

4.9.5 Flow cytometry detection of CD4 and CCR5 expression

CMCs obtained from the subjects were stained with anti-CD3, anti-CD4 and anti-CCR5 monoclonal antibodies and then analyzed by flow cytometry for the expression levels of these cell surface glycoproteins. Briefly, the cells were stained in a total volume of 90µl of the fluorochrome conjugated monoclonal antibodies in FACS buffer. After 30 min incubation in the dark at 4^0C, the cells were washed and fixed using 1% paraformaldehyde. The stained cells were analyzed using FAScalibur software in an LSRII machine.

4.10 QUALITY ASSURANCE CONSIDERATIONS

Sample collection was done by well trained nurses and technicians at the clinic. The samples were then transported in a cool box to UNITID laboratories. The samples were processed under sterile environment following the standard operating procedures for each test that were performed. All the required equipment for the performance of the assays which included safety cabinets, a real time PCR machine, a thermocycler, a gel electrophoresis tank and an LSR II machine were available at UNITID and were well calibrated and serviced annually.

4.11 DATA MANAGEMENT

A nurse counselor and designated laboratory technologists collected both the demographic data and study samples from the recruited subjects. The demographic data was entered on to designed data collection forms (DCFs) and log books. A unique study number was designated to each recruited subject for identification on the DCF. Any other information for the purpose of tracing the study subjects such as names, address, and phone numbers were kept separate and confidential. The collected data was later entered onto a designed database for the study. This data was used in data analysis upon completion of the laboratory procedure.

Data analysis was done using three statistical tests; Pearson's Coefficient Correlation test, Analysis of Variance (ANOVA) and Tukey's post test. Pearson's test was used to determine the correlation between HHV7 infection and surface expression of the $CD4^+$ T cell receptors. ANOVA was used to determine the significance in the variations of the means obtained across all the study groups while Tukey's pos`t test was used to determine the mean different significance in between groups. Flow cytometry data was analyzed using Flow Jo software, Version 10.0.7 (Tree Star, Inc). This is a statistical package that is used to analyze FCS (flow

cytometry data file format standard) files generated by FACS DIVA software on LSR II machine. The Flow Jo software gave the expression levels (median fluorescent intensity (MFI)) and the frequencies of the target markers in the samples. The generated data on Flow Jo was then exported to Graph Pad Prism 5 software for correlation, comparison and significance analysis using the mention statistical tests. Both RT PCR and conventional PCR data was also analyzed using Graph Pad Prism 5 software. The level of significance was determined and annotated using asterisks as guided by the Graph Pad Prism scheme; table 6:

Table 6: Level of Significance Scheme

P value	Wording	Summary
<0.0001	Extremely significant	****
0.0001 to 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
≥0.05	Not significant	ns

5.0 RESULTS

5.1 HHV7 PREVALENCE AND VIRAL LOADS IN THE STUDY POPULATION

Out of the 125 study subjects recruited, 2 male subjects did not bring their semen samples and two other semen samples were contaminated during laboratory processing and so could not be analyzed. The analyzed 121 subjects had a mean age of 40 years for males and 35 years for females. The prevalence of HHV7 in the study subjects was found to be 26%. Of the 26%, males were 53% and females were 47% (figure 1a and table 7). The detection of HHV7 was done using TaqMan RTPCR as illustrated in figure 1b.
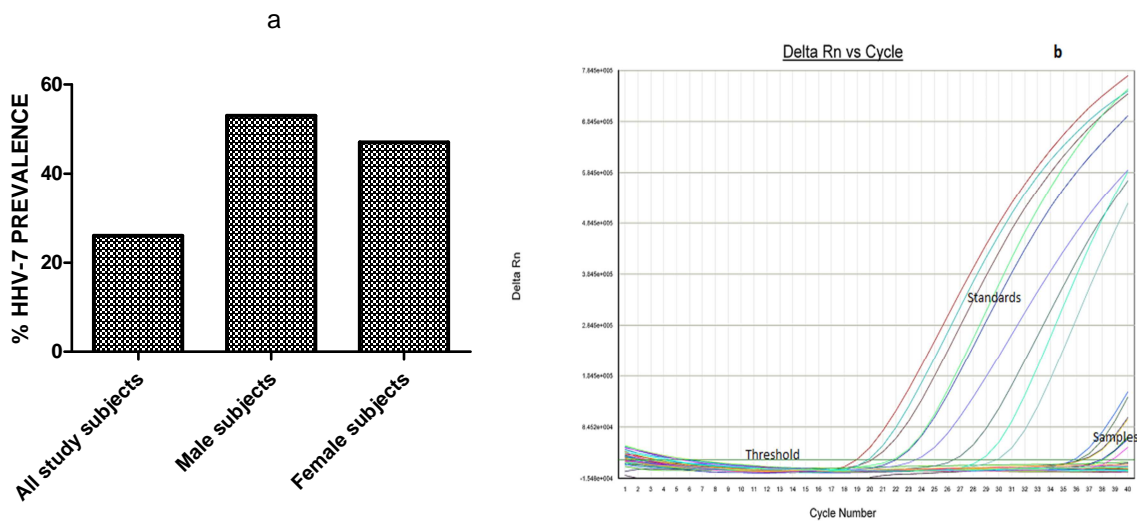

Figure 1: HHV7 prevalence in the study subjects (1a) and the RTPCR plot (1b).

Table 7: HHV7 Prevalence

|	Males	Females	Total
General Prevalence			
HHV7 Positive	17	15	32
HHV7 Negative	39	50	89
Total	56	65	121
% Prevalence	30	23	26
Mean Age (years)	40	35	37
HIV Positive Prevalence			
HHV7 Positive	6	5	11
HHV7 Negative	16	20	36
Total	22	25	47
% Prevalence	27	20	23
HIV Negative Prevalence			
HHV7 Positive	6	5	11
HHV7 Negative	18	20	38
Total	24	25	49
% Prevalence	25	20	22
CONTROLS (All HIV-) Prevalence			
HHV7 Positive	5	5	10
HHV7 Negative	5	10	15
Total	10	15	25
% Prevalence	50	33	40

Similar to the prevalence, males had a significantly higher HHV7 viral load in comparison to the female subjects (figure 2A). When separated according to the HIV infection status, the HIV+ subjects had slightly higher but not significant HHV7 viral loads than in the HIV negative subjects. Male subjects still showed significantly higher HHV7 viral loads despite their HIV infection status as compared to female subjects (figure 2C and 2D).

A

HHV-7 (cp/ml)

p=0.0001
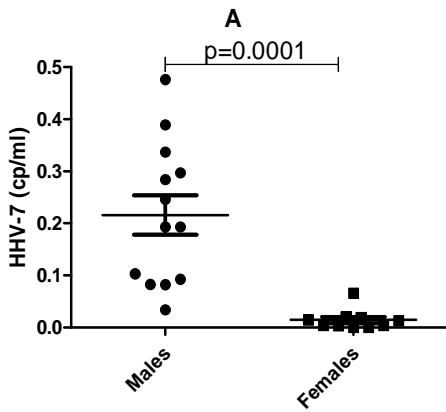
Males   Females

On average, male subjects had significantly higher HHV7 viral loads (0.22cp/ml) than female subjects (0.01cp/ml)

B

HHV-7 (cp/ml)

p=0.98

HIV+VE   HIV-VE
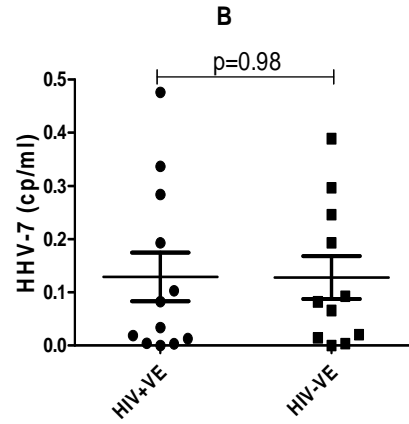
Based on their HIV infection status, there was no significant difference in the HHV7 viral loads between the HIV positive subjects and HIV negative subjects.

C

HHV-7 (cp/ml)

p=0.02

HIV+ Males   HIV+ Females
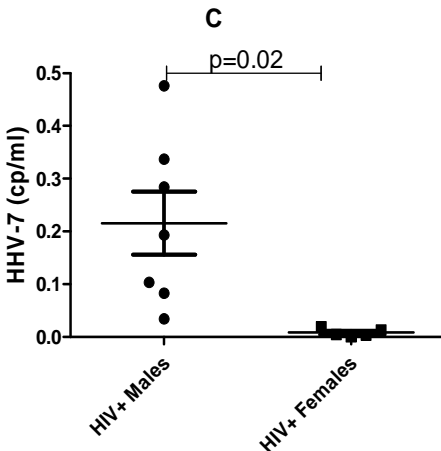
HIV positive male study subjects had significantly higher HHV7 viral loads as compared to HIV positive female study subjects

D

HHV-7 (cp/ml)

p=0.006

HIV- Males   HIV- Females
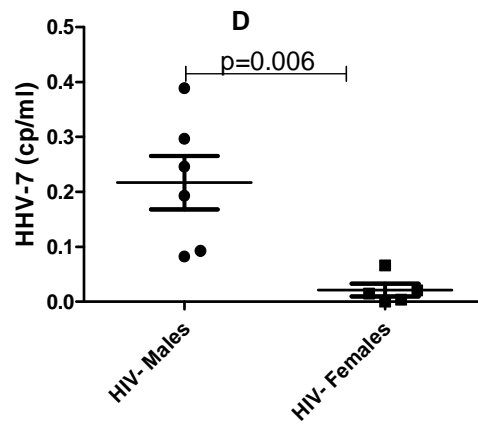
HIV negative male study subjects had significantly higher HHV7 viral loads when compared to female study subjects

Figure 2: HHV7 viral loads according to gender and HIV status
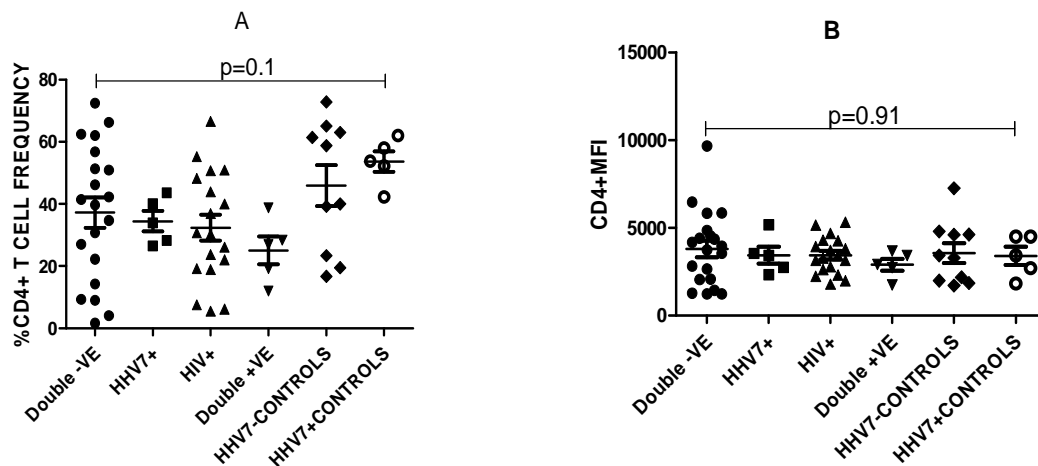
5.2 EFFECTS OF HHV7 INFECTION ON CD4+ T CELL FREQUENCY AND EXPRESSION OF CD4 AND CCR5 SURFACE RECEPTORS IN HIV POSITIVE AND HIV NEGATIVE STUDY SUBJECTS

The control study subjects had the highest CD4+ T cell frequency with those that were HHV7 positive having a higher frequency than the HHV7 negative controls. In the discordant couple study subjects, the highest CD4+ T cell frequency was observed in those that were both negative for HHV7 and HIV (double negative). The lowest frequency was observed in discordant couples that were both positive for HHV7 and HIV infection (figure 3A). Reduced expression of the CD4 receptors was observed in the HHV7 positive controls as compared to the HHV7 negative controls. Similarly, reduced CD4 expression was observed in HHV7 positive discordant couples in comparison to the double negative discordant couples (figure 3B). Unlike the CD4 receptors, CCR5 receptor expression was higher in HHV7 positive, HIV positive and HHV7/HIV co-infected discordant couples as compared to the double negative couples. In the control study subjects however, CCR5 receptor expression was lower in the HHV7 positive controls as compared to the HHV7 negative controls (figure 3C).

The control study subjects had the highest %CD4+ T cell frequencies while the HIV positive discordant couples had the lowest %CD4+ T cell frequencies

A reduction trend in the expression levels of the CD4 receptor was observed in all HHV7 positive study subjects

C
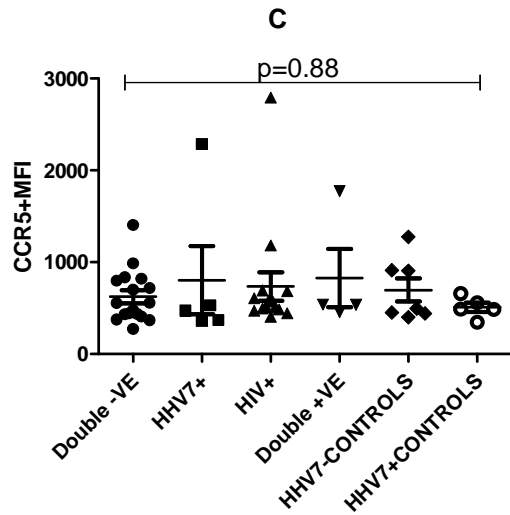
Both HHV7 and HIV infected discordant couple study subjects had increased expression levels of the CCR5 receptor. However, the HHV7 positive controls had reduced CCR5 expression levels when compared to the HHV7 negative controls

Figure 3: Effects of HHV7 Infection on HIV Receptors

5.3 CORRELATION BETWEEN HHV7 VIRAL LOADS AND EXPRESSION OF HIV RECEPTORS (CD4 AND CCR5)

A significantly positive correlation was observed between HHV7 viral load and CD4+ T cell frequency in all the study subjects (figure 4A(i)). A strong positive correlation was also observed in the HIV negative discordant couples (figure 4A(ii)). On the contrary, the surface expression level of the CD4 receptors was negatively correlated to the HHV7 viral loads and this was stronger in the HIV negative discordant couples (figure 4B). Similar to the CD4 MFI, the surface expression levels of the CCR5 receptors had a negative correlation with the HHV7 viral loads (figure 4C).

27
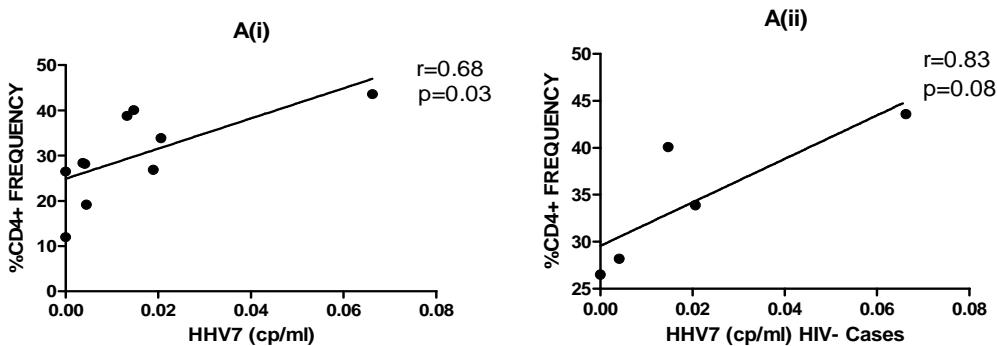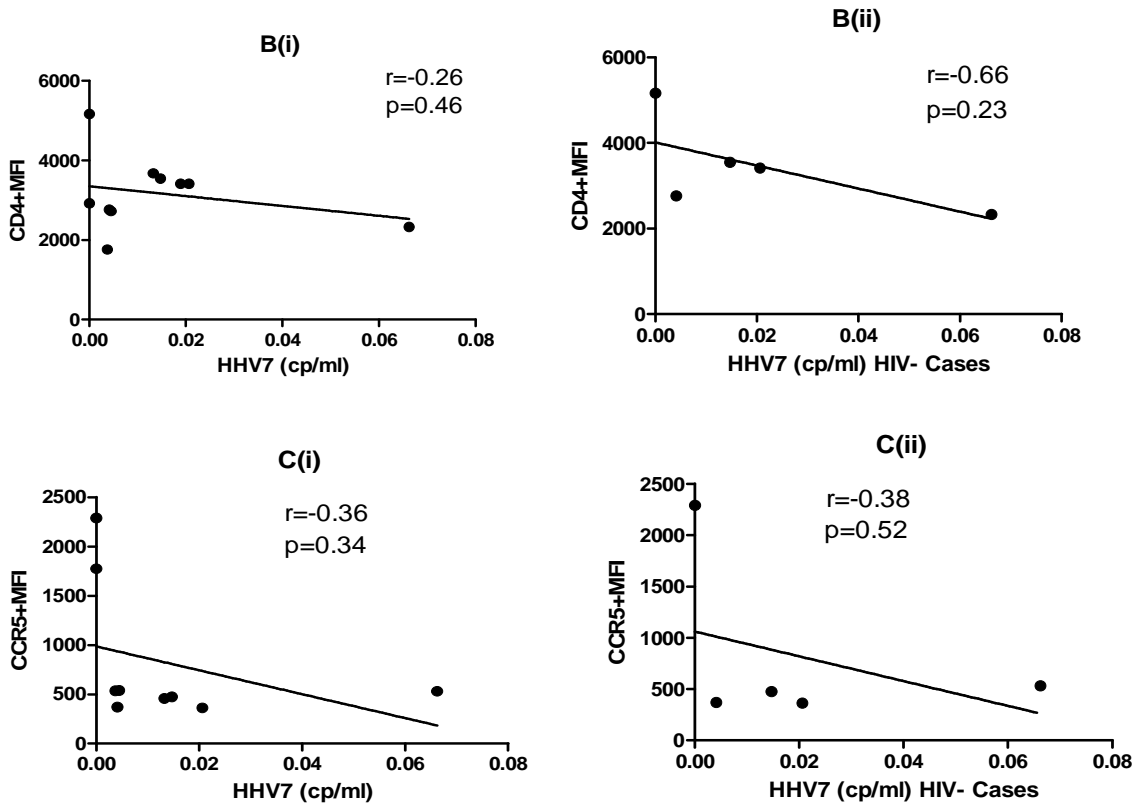
Figure 4: Correlation between HHV7 viral loads and %CD4+ T cell frequency in (Ai) all study subjects and (Aii) in HIV negative discordant couples. CD4 and CCR5 MFI in (Bi and Ci) all study subjects and (Bii and Cii) in HIV negative discordant couples.

5.4   CONVENTIONAL PCR RESULTS

Conventional PCR was performed to detect cell associated HHV7 genome. Apart from cases that were both positive for HHV7 and HIV, increased CD4+ T cell frequencies were observed in all HHV7 positive subjects in comparison to the HHV7 negative subjects (figure 5Ai). The HHV7 positive controls had the highest CD4+ T cell frequency and this was significant as observed when compared to all HHV7 negative discordant couples regardless of their HIV infection status (figure 5Aii). Similarly, HHV7 positive controls had significantly higher CD4+ T cell frequency when compared to HHV7/HIV co-infected discordant couples and also in comparison to the discordant couples neither infected with HHV7 nor HIV (figure 5Aiii and 5Aiv).

The expression of the CD4 antigen was however lower in all the HHV7 positive discordant couples while higher in the HHV7 positive controls as compared to the HHV7 negative study

subjects (figure 5B). On the other hand, higher CCR5 expression levels were observed in all HHV7 positive subjects (figure 5C).

A(i)
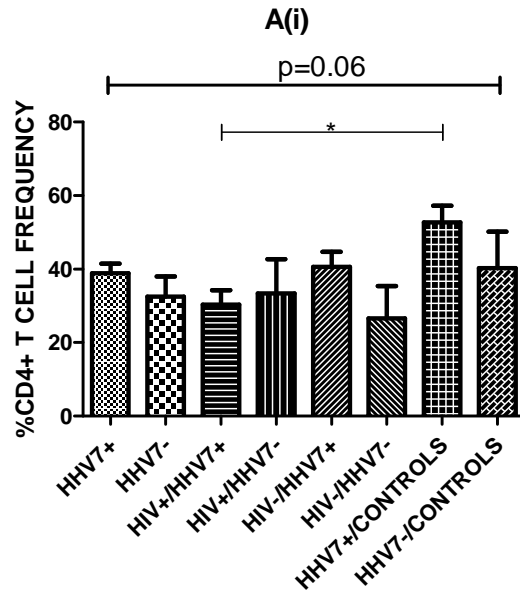
Apart from the HIV positive discordant couples, all study subjects that were positive for HHV7 had higher %CD4+ T cell frequencies as compared to study subjects that were HHV7 negative. HHV7 positive controls had the highest %CD4+ T cell frequency and this was significantly higher when compared to the HIV positive discordant couples that were also positive for HHV7.

A(ii)
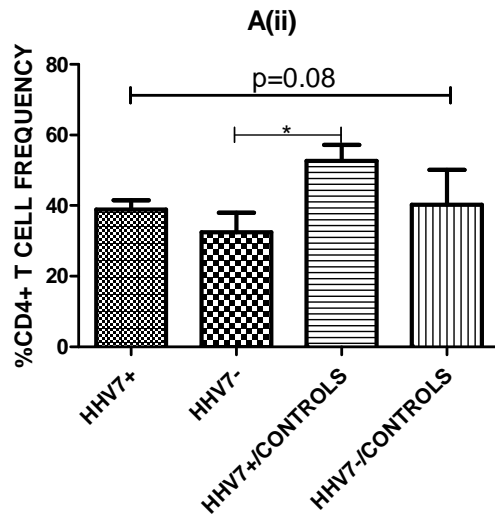
On comparing the controls versus the discordant couples, the HHV7 positive controls had significantly higher %CD4+ T cell frequency than the HHV7 negative discordant couples.

A(iii)
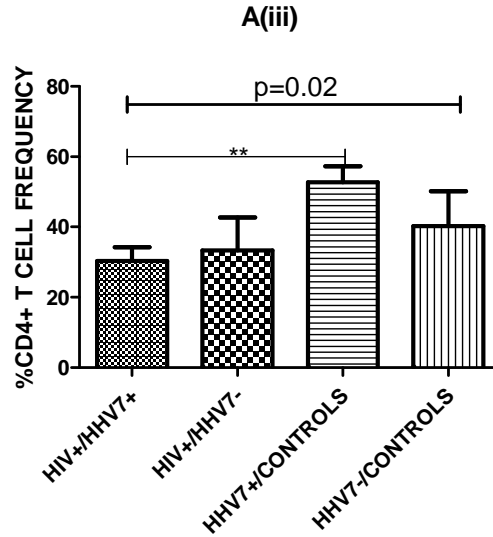
There was an overall significant difference (p=0.02) in the %CD4+ T cell frequencies across the study subjects. In addition, the HHV7 positive controls had a significantly higher %CD4+ T cell frequency than the HIV+/HHV7+ discordant couples.

A(vi)
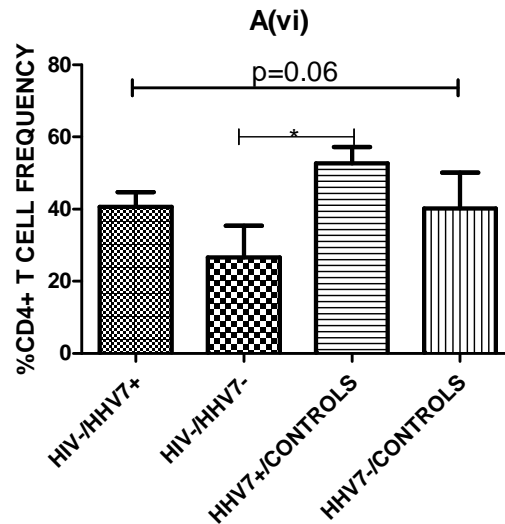
HHV7 positive study subjects both in the HIV negative discordant couples and the controls had higher %CD4+ T cell frequencies than the HHV7 negative study subjects. A significant difference was observed between the HHV7 positive controls and the HHV7 negative/HIV negative discordant couples.

B
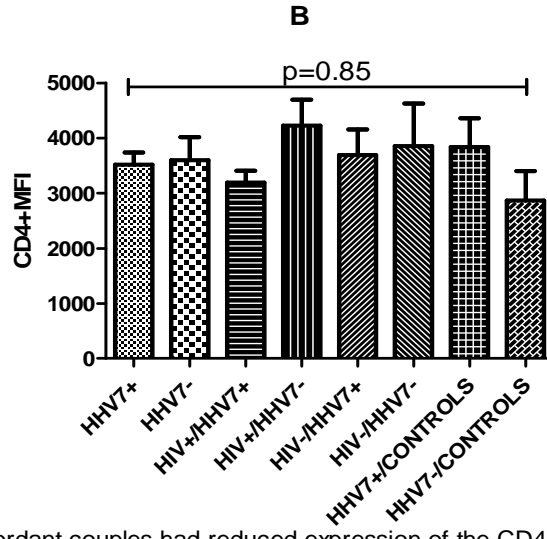
All HHV7 positive discordant couples had reduced expression of the CD4 receptor. However, the HHV7 positive controls had a higher CD4 receptor expression than the HHV7 negative controls

C
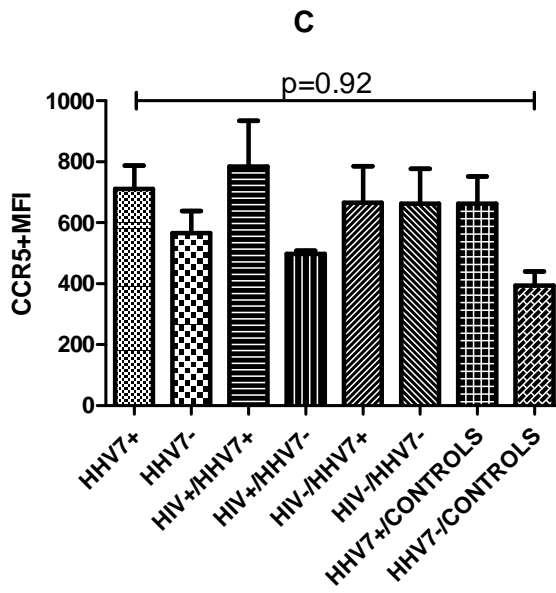
All HHV7 positive study subjects had higher expression of CCR5 receptors compared to the HHV7 negative study subjects

Figure 5: Conventional PCR results: (A) %CD4+ T cell frequency, (B) CD4 MFI and (C) CCR5 MFI

6.0   DISCUSSION

A study by Laman and his colleagues in 2014 showed that HHV7 infection may occasionally result in severe disease and death (Laman et al. 2014). However, they stated that it may be difficult to demonstrate the causal relationship between the viral nucleic acid presence and the clinical disease (Laman et al. 2014). A review by Rivka C. Stone showed that HHV7 infection was associated with seizures in children but still indicated that it was unclear whether these febrile seizures were as a result of a direct viral invasion into the central nervous system or as a result of escalating fever from the viral infection (Stone, Micali, and Schwartz 2014). This was due to the infrequent detection of the viral nucleic acid in the cerebrospinal fluid. Both studies indicate HHV7 as a potential pathogenic virus. However, like other roseoloviruses, HHV7 has been shown to undergo latency upon infection and is most commonly only reactivated in immunocompromised situations (Magalhães et al. 2011; Stone, Micali, and Schwartz 2014). This could therefore indicate that HHV7 may not be pathogenic.

This study assessed the link between HHV7 infection and resistance to HIV infection. As per Wolz and his colleagues in 2012, HHV7 has a nearly universal prevalence (Wolz, Sciallis, and Pittelkow 2012). However, mode of HHV7 transmission is yet to be described since the salivary system is the only source currently perceived to produced infectious HHV7 (Minarovits, Gonczol, and Valyi-Nagy 2006). However, from this study results, it is possible that HHV7 could also be sexually transmitted due to the isolation of the virus from semen and cervical mucosa samples.

Previous studies have shown HHV7 induced loss of CD4 antigens on T lymphocytes upon infection rendering these cells resistant to HIV-1 infection (Paola Secchiero et al. 1998; Lisco et al. 2007). However, these studies were done *in-vitro* and it has not been shown whether or not this is the case *ex-vivo*. This study compared the *ex-vivo* CD4+ T cell frequencies, CD4 and CCR5 antigen expression between HHV7 infected and non infected HESN population. From the general HHV7 prevalence of 26%, males had 53% prevalence and females 47% prevalence. Similarly, males had a significantly higher HHV7 viral loads as compared to females despite the HIV status (p=0.02 in HIV positive study subjects and p=0.006 in HIV study subjects). This could be attributed to the stronger immune responses in females as opposed to males who produce higher levels of testosterone which is immunosuppressive (Furman et al. 2014). It is therefore possible that the female cases had cleared most of the HHV7 from their bodies.

The HHV7 positive subjects in the control group had the highest CD4+ T cell frequency. However, contrary to our expectation due to the control situation, the HHV7 positive subjects in the discordant couples group had a slight reduction in the CD4+ T cells frequency in comparison to their counter parts who were both negative for HHV7 and HIV. In the conventional PCR result, high CD4+ T cell frequencies were observed in all HHV7 positive study subjects apart from the HIV positive discordant couples who had a lower CD4+ T cell frequency in comparison to the HHV7 negative subjects. These results supports a study by Lisco and his colleagues in which they observed a slight reduction of the CD4+ T cells in HHV7 infected tissues during productive infection (Lisco et al. 2007). This could therefore explain the reduction in the CD4+ T cells in the HHV7 positive discordant couples that were either HIV positive or exposed to the HIV due to the frequent invasion of the HIV from their HIV positive partners. Generally, the control population had the advantage of not being under the HIV induced depletion of the CD4+ T cells. The increased CD4+ T cells in the HHV7 positive control group could also be beneficial to them in terms of offering protection against other infections. In addition, the control population had significantly high CD4+ T cell frequency when compared to the HIV positive discordant couples. This was expected as HIV is known to deplete the CD4+ T cells. Furthermore, from the correlation graphs, HHV7 viral loads were observed to have a strong positive correlation with CD4+ T cells.

Similar to observations made in previous HHV7 studies (Lusso et al. 1994; Lisco et al. 2007), this study observed reduced surface expression of the CD4 antigens on the CD4+ T cells of HHV7 positive study subject. On correlating the HHV7 viral loads to the CD4+ T cell frequencies, a strong positive and significant correlation was observed in the general population regardless of the HIV status (r=0.68, p=0.03). A much stronger positive correlation was observed in the HESN population (r=0.83, p=0.08). Contrary to this, the CD4 antigen surface expression levels were negatively correlated to the HHV7 viral loads both in the general population and in the HESN group (r=0.26 and r=0.66 respectively). This negative correlation further supports the previous study by Lusso and his colleagues in 1994 where they observed a progressive down regulation of surface CD4 antigens on normal human peripheral CD4+ T cells infected with HHV7 for 9 days (Lusso et al. 1994). Similar negative correlations were observed between HHV7 viral loads and expression of CCR5 surface antigens. However, a study by Yasukawa and his colleagues showed that CCR5 antigens were unnecessary for HHV7 infection into the CD4+

T cells and there was no down regulation of these receptors upon infection of these cells with HHV7 (Yasukawa et al. 1999). This therefore means that other factors could have played a role in the negative correlation between the HHV7 viral loads and the CCR5 expression levels.

7.0   CONCLUSSION

Our study results show a similar trend to studies that have been done in vitro on HHV7 effects on the number of CD4+ T cells and there receptor expression. These results could therefore mean that HHV7 provides protection to the HESN population through the reduction of the CD4+ T cells hence reduce the target cells for HIV and the reduced surface expression of the CD4 antigens hence interfere with HIV infection. In the control population where the cells are not activated due to absence of HIV, we speculate that HHV7 infection and development into latency could be advantageous by increasing the number of immune cells (CD4+ T cells). This therefore increases protection of this population against any other infections.

8.0   RECOMMENDATIONS

Similar trends, although in some cases not significant, were observed between this *ex-vivo* study and previous *in-vitro* studies. A strong positive correlation between HHV7 viral loads and CD4+ T cells frequencies was observed. In contrast, a negative correlation was observed between HHV7 viral loads and CD4 receptors. It would therefore be of interest to investigate whether having high numbers of CD4+ T cells with reduced expression of the CD4 receptors have any effect on the overall immunity. It would also be of interest to investigate the exact mechanism by which HHV7 interferes with the transcription of the CD4 receptors.

More *ex-vivo* studies on larger sample sizes would also give a clear validation of the HHV7 induced reduction in the expression of the CD4 receptors. A longitudinal study would enable monitoring the trends between CD4+ T cell frequencies with their receptor expression and HHV7 viral loads and the correlation of HHV7 viral loads with HIV viral loads. All these studies may guide in the search for an effective HIV intervention.

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APPENDICES

APPENDIX 1: INFORMED CONSENT FORM

Performance site: Majengo-Pumwani clinic, Nairobi

Names of the investigators:

    a) Akiso Matrona Mbendo (Principal Investigator)

       University of Nairobi, Center for Biotechnology and Bioinformatics,

       E-mail: Matakiso@yahoo.com

    b) Dr. Julius Oyugi (Supervisor)

       MSc, PhD, Senior Lecturer,

       Department of Medical Microbiology,

       University of Nairobi

       E-mail: julias_oyugi@hotmail.com

    c) Prof. James O. Ochanda (Supervisor)

       MSc, PhD, Director,

       Center for Biotechnology and Bioinformatics (CEBIB),

       University of Nairobi

       E-mail: jochanda@uonbi.ac.ke

Sponsor: Self sponsored

My name is Akiso Matrona Mbendo. I am a postgraduate student at the University of Nairobi and I would like to invite you to participate in this study. The information on this document is meant to help you make a decision on whether or not to participate in the study. Kindly feel free to ask any questions or raise your concerns.

Introduction: Human Immunodeficiency Virus (HIV) is the causative agent of Acquired Immunodeficiency Syndrome (AIDS). This virus targets and kills the cells of your body that provide defence to other infecting agents. By killing these cells HIV weakens your ability to

fight diseases which then lead to death. However, another virus called Human Herpes Virus 7 (HHV-7) also infects the same cells as HIV but does not kill them. This virus may prevent HIV from infecting the same cells and therefore protect humans from infection with HIV. It is still not known whether this is true or not. If found to be true, the knowledge gained from this study may be useful in formulating products that can be used to protect or prevent transmission of HIV between humans.

The Purpose of the Study: To establish whether HHV-7 can prevent HIV from infecting humans and therefore protect them from such infections.

Procedure: The study will take one visit; during the first visit, study samples will be collected at the clinics and the samples transported to UNITID in a cool box for laboratory procedures.

Study Entry Requirements: The study will recruit adults aged between 18 and 50 years, and not pregnant. During the visit, you will be at the study clinic for about 20 minutes. If you agree to join after being fully informed on the study and any of your questions answered to your satisfaction, you will sign or mark 2 copies of the Informed Consent Form. This is to confirm that you have been informed about the study and voluntarily agree to take part. One copy is yours to keep and the other will be kept in our confidential file. If you do not wish to keep your copy, you will sign or mark a form that states you do not want to take it, and we will keep it for you. You will be asked questions about your general health and medical examination will be performed by one of the trained nurses at the clinic.

The following specimens will be collected: a) blood: Ten (10) mls of venous blood will be collected directly into a 10ml EDTA vacutainer tube using a vacutainer needle. b) Cervical mononuclear cells (CMCs) will be collected using a cytobrush which will be gently inserted into the cervix and rotation 360^0. c) Cervical vaginal lavage (CVL) will be collected by washing the cervix using 2ml $1\times$ phosphate buffered saline and drawing the fluid using a syringe. A speculum will be used in the collection of both the CMCs and CVL. You will be given a d) urine collection cup into which you will collect approximately 10mls of urine and then seal with the cup lid. e) semen samples will be collected from male study participants only. The participants will be given a condom into which they will collect semen during sexual intercourse with their partners.

The specimen will then be brought to the clinic the following day in the morning. **All the specimens will be used for the intended study only.**

Risk of Participation: Risks of participating in this study are minimal. There may be some risks involved in drawing blood but these are uncommon. However, you may have pain and bruising where the needle goes into your arm. You may feel dizzy or faint. There are no anticipated physical risks in participating in this study. However if there are any injuries that may arise due to your participation, you will be offered treatment by the study doctor free of charge. Cervical lavage procedures are carried out with an experienced nurse and using sterile speculum (disposable) and phosphate buffered saline. The cervical swabs used for CMCs collection are also sterile. The specula and the swabs are used separately for each participants hence there is no cross infection in any case. There are possibilities of experiencing some pain as the speculum is inserted in the cervix. However, a sterile gel is used to avoid this pain.

Anticipated Benefits: There are no direct benefits to you. However, if the results of this study are positive, it may lead to the developing of new treatment for HIV.

Injuries: We do not expect you to be injured as a result of being in this study. If you are hurt as a result of being in this study, the clinic staff will give you the necessary treatment for your injuries including emergency treatment for free. You will not have to pay for this treatment. You will not receive any money or other form of payment for such injuries. You will not give up any legal rights by signing this consent form.

When can you leave the Study: Your participation in this study is completely up to you. You can leave this study at any time without giving a reason. Withdrawal will NOT compromise any rights you had before entry into the study or influence any current or future medical care you may need. You may be removed from the study without consent for the following reason: If the study is stopped or cancelled. If you leave or are asked to leave the study after lab tests have been done, you may still get your test results from the study site.

How much will it Cost: You do not have to pay to be in the study.

Confidentiality: Your participation in the study, all information collected about you, and all laboratory test results will be available to no one except the study team. You will be identified only by your own unique identity number, which is known only by you and the clinic staff. Apart from the team members that you meet, other staff from National or International government regulatory agencies, members of the Ethics Committee, study monitors, auditors, inspectors, and representatives of the Sponsor may check the records to make sure the study was conducted properly. They are equally bound to respect your confidentiality. Your identity will not be disclosed in any publication or presentation of this study.

Contact Numbers: If you have any questions regarding the study of your participation in the study, you can call **Akiso Matrona Mbendo,the Principal Investigator, Mobile No: 0724818591 or Dr. Julius Oyugi, Co-Investigator, Mobile No: 0713898564**.

If you have a question about your rights as a research volunteer you should contact **Prof. Guantai, the chairman of the Ethics Committee of Kenyatta National Hospital, Tel: (+254-020) 276300 ext 44355 or email uonknherc@uonbi.ac.ke**

APPENDIX 2: INFORMED CONSENT DOCUMENT

I, (name of volunteer)………………………………………………………………………………

Of (address)………………………………………………………………………………………

Agree to take part in the research project entitled: **Association between HHV-7 Infection and Resistance to HIV-1 Infection among HIV-1 Resistant Persons**. I have been told in detail about the study and know what is required of me. I understand and accept the requirements. I understand that my consent is entirely voluntary and that I may withdraw from the research study for any reason, and this will not affect the legal rights I may otherwise have. My questions have been answered to my satisfaction.

Participant: Print Name: …………………………………………………………………..

Signature/Mark/Thumbprint: …………………………………………………………………..

Date: (DD/MM/YYYY)   ……./……./……….

Person Obtaining Consent:

I have explained the nature, demands and foreseeable risks of the above study to the volunteer and answered his/her questions:

Print Name: …………………………………………………………………………………..

Signature: …………………………………………………………………………………

Date: (DD/MM/YYYY)   ……./……./……….

Impartial Witness: (*only necessary if volunteer was not able to read and understand the Consent Information Sheet and Informed Consent Document*):

I affirm that the Informed Consent Document has been read to the volunteer and he/she understands the study, had his/her questions answered, and I have witnessed the volunteer's consent to study participation.

Print Name: ………………………………………………………………………………….

Signature/Mark/Thumbprint: ………………………………………………………………….

Date: (DD/MM/YYYY)   ……./……./……….

APPENDIX 3: QUESTIONNAIRE

Volunteer ID:………………………..

Date of Interview: (DD/MM/YYYY)   ……./……./……….

1. Demographic information

a. Date of Birth: (DD/MM/YYYY)   ……./……./……….

b. Sex: Male ☐   Female ☐

c. What is your marital status? Married ☐   Single ☐   Divorced ☐   Widowed ☐

d. If married, when did you get married?   (Year) …………..

e. Is this your first marriage?   Yes ☐   No ☐

   If the answer to question (e) above is NO, answer the following the question

f. How many times have you been married?   Once ☐   Twice ☐   Thrice ☐

        More ☐   Specify:………………

2. HIV status

a. Do you know your HIV status?   Yes ☐   No ☐

b. What is your HIV status?   Positive ☐   Negative ☐

c. If your HIV status is positive, when were you first tested positive for HIV? ……..(Year)

d. If your HIV status is negative, when were you last tested for HIV? …………….(Year)

3. Laboratory Results

HIV status: Negative ☐   Positive ☐