HIV CO-INFECTIONS WITH CYTOMEGALOVIRUS, HEPATITIS C VIRUS AND HUMAN PAPILLOMAVIRUS IN NORTHERN SOUTH AFRICA

BY

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DECLARATION

I Rikhotso Mikateko, hereby declare that the dissertation for Master of Science degree in Microbiology at the University of Venda, hereby submitted by me, has not been submitted before for the degree at this or any other university, this is my original work in design and execution, and that all the reference materials contained therein have been duly acknowledged.

Student RMhotso Date 30/04/14

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DEDICATION

I would like to dedicate this work to my two lovely sons, **Tshembo** and **Theothomas Ngobeni**. They are the reason I kept on doing research and studying furthermore. Even though I spent less time with them, I appreciate their patience and tolerance of my absence in their childhood. Therefore, they deserve to be honored by this work.

ABSTRACT

The human immunodeficiency virus (HIV) is responsible for acquired immunodeficiency syndrome (AIDS) affecting millions of people globally. Opportunistic pathogens, such as human papillomavirus (HPV), cytomegalovirus (CMV) and hepatitis C virus (HCV) are associated with HIV infection and AIDS.

Cervical cancer caused by HPV remains a serious public health problem in Sub-Saharan Africa and many other parts of the developing world. The prevalence of HPV is higher in African women with a normal cervical cytology than in women in other regions of the world. Although HPV prevalence data is accumulating in several regions of South Africa, there is no data on the HPV infection in northern South Africa.

Cytomegalovirus is the leading cause of congenital infections. Risk of congenital infection is higher for seronegative women who have a primary CMV infection during pregnancy than it is for seropositive women who experience a reactivation of re-infection. Hepatitis C virus causes liver disease and is a major public health problem for people infected with HIV. Data on HCV prevalence is equally scarce for northern South Africa.

The aim of the study was to determine the prevalence of CMV, HPV and HCV, and also to determine the genotypes of HPV in Northern South Africa in an HIV infected population. The study subjects (n=200) comprised highly active antiretroviral therapy (HAART) naïve and experienced individuals of both genders. The age range was 2-68 years. This was a laboratory based retrospective study. The 200 HIV positive plasma samples were screened for HPV, CMV and HCV antibodies by enzyme-linked immunosorbent assay (ELISA). A group of 50 HIV negative samples was included in the detection of the co-infecting viruses. HPV DNA detection from plasma and peripheral blood mononuclear cells (PBMCs) was done by PCR and genotyped by reverse hybridization using the Linear Array HPV Genotyping Test kit (Roche). Genotyping was further confirmed by phylogenetic analysis of a partial L1 gene.

The antibody prevalence of HPV, CMV and HCV in the HIV infected population was 21% (42/200), 100% (200/200) and 0.05% (1/200) respectively. In the HIV negative population the prevalence was 12% (6/50), 100% (50/50) and 0% (0/50) respectively. Infection with CMV was significantly more prevalent among the unmarried patients than married patients (p=0.000). Infection rate with HPV was not significant in HIV infected females (p=1.000) when compared with the HIV negative group. Similarly, there was no significant difference of HPV infection when age below 15 years and above was considered in the HIV infected and non-infected groups (p=0.983). Forty two (21%) of HIV infected people were found to be co-infected with both HPV and CMV.

Amplification of HPV DNA from HPV plasma could not yield expected DNA fragment (450bp). However expected DNA was obtained from 50% (8/16) PBMCs of the corresponding plasma. Genotyping by Linear Array HPV Genotyping Test was successful for 50% (4/8) of the HPV DNA and all were of the HPV type 16. Ten samples, successfully amplified by conventional PCR out of 16, were shown to be HPV type 16 by phylogenetic analysis.

The study findings show that a low percentage of the study population was infected with HPV, CMV and HCV. In addition, infection with HCV was also low. The limited genotyping showed that HPV type 16 is the more prevalent variant in the studied population. These observations would require confirmation with a larger sample size and HPV genotyping on other specimen type such as cervical cells.

LIST OF ABRREVIATIONS

AIDS – Acquired immunodeficiency syndrome

ABC – Abacavir

AZT – Zidovudine

AAHS – Amorphous aluminum hydroxysulfate

ARVs – Antiretrovirals

CMV – Cytomegalovirus

CCR5 – Chemokine receptor 5

cDNA – Complementary Deoxyribonucleic acid

CD4+ - Cluster of differentiation

CXCR4 – Chemokine receptor 4

DNA – Deoxyribonucleic acid

ddI – Didanosine

d4T – Stavudine

E1 – Early gene 1

E2 – Early gene 2

EFV – Efavirenz

EIA – Enzyme immuno assay

ELISA – Enzyme-linked immunosorbent assay

ENV – Envelope

FDA – United States Food and Drug Administration

FIs – Fusion Inhibitors

FTC – Emtricitabine

Gag – Group associated antigen

HAART – Highly active antiretroviral therapy

HCV – Hepatitis C virus

HHV5 – Human herpesvirus 5

HIV – Human immunodeficency virus

HPV – Human papillomavirus

IN - Integrase

INIs – Integrase inhibitors

LEEP – Loop electrosurgical excision procedure

L1 – Late gene 1

L2 – Late gene 2

mRNA – messenger Ribonucleic acid

MPL – MonophosphorylInlipid

NAT – Nucleic acid based test

Nef – Negative regulatory factor

NES – Nuclear export signal

NLS – Nuclear localizing signal

NRTIs – Nucleoside reverse transcriptase inhibitors

NNRTIs – Non-nucleoside reverse transcriptase inhibitors

NVP – Nevirapine

OD – Optical density

PBMCs – Peripheral blood mononuclear cells

PCR – Polymerase chain reaction

PIC – Pre-integration complex

PIs – Protease inhibitors

Pol – Polymerase

Rev – Regulaor of expression of virion

RIBA – Recombinant immunoblot assay

RNA – Ribonucleic acid

SIVcpz – Simian immunodeficiency virus chimpanzee

SIVsm – Simian immmunodeficiency virus sooty mangabeys

TAR – Transactivation response element

Tat – Transcriptional trans-activator

TB – Tuberculosis

TDF – Tenofovir

3TC – lamivudine

UNAIDS – Joint United Nations Programme on HIV/AIDS

UL – Unique long

Vif – Viral infectivity factor

Vpr – Viral protein R

Vpu – Viral protein U

WHO – World health organization

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1. INTRODUCTION

Acquired immunodeficiency syndrome (AIDS) is a disease caused by the human immunodeficiency virus (HIV). This condition progressively reduces the effectiveness of the immune system and leaves individuals susceptible to opportunistic infections and tumors. In 2012, there were 35.3 million people infected with HIV worldwide of which 2.3 million were new infections and 1.6 million people died from AIDS-related illnesses worldwide. In Sub-Saharan Africa, people who were infected with HIV in 2012 were 25 million and 5.38 million in South Africa (UNAIDS, 2013). In 2011, there were 29,5% of pregnant women aged 15-49 years infected with HIV attending the antenatal clinics in South Africa. KwaZulu-Natal had the highest HIV prevalence followed by Mpumalanga, Free State and North West while Northern and Western Cape had the lowest prevalence (Department of Health, 2012).

Many people living with HIV are co-infected with the hepatitis C virus (HCV). Hepatitis C is an infectious disease that primarily affects the liver, caused by the hepatitis C virus. HCV co-infection rates are the highest among persons who have acquired HIV from injecting drug use. Hepatitis C is a major public health problem for people living with HIV. Persons chronically infected with the same HCV genotype are likely to respond to the same dose and duration of peginterferon and ribavirin therapy if they are HIV co-infected. People with HIV co-infection with HCV are likely to develop cirrhosis over a shorter period of time (www.who.int/csr/disease/hepatitis/world_hepatitis).

Human cytomegalovirus (CMV) is a ubiquitous agent that commonly infects individuals from diverse geographical and socio-economic backgrounds. CMV remains the leading

cause of congenital viral infection and a significant cause of transfusion-acquired infections in the immunocompromised (Akinbami et al., 2009).

CMV causes retinitis that leads progressively to complete blindness without treatment. CMV retinitis is one of the most common opportunistic infection in persons with AIDS, typically those with CD4+ lymphocyte counts below 50 cells/μL. Human cytomegalovirus infection is typically unnoticed in healthy people, but can be life-threatening for the immunocompromised such as HIV-infected persons, organ-transplant recipients, or new born infants.

Human Papillomavirus (HPV) infections are diagnosed worldwide and account for the most common sexually transmitted disease worldwide. HPV is the major cause of cervical cancer in women of child-bearing age. Sexually active women have a lifetime risk of up to 80% to be infected with one or more HPV types. In South Africa, there is a highly incidence of cervical cancer among South African women, with an age-standardized incidence rate of 30.5 per 100,000 per year (Marais et al., 2008).

1.2. LITERATURE REVIEW

1.2.1. THE HIV LIFE CYCLE

The HIV life cycle begins with the viral gp120 binding to the host cell CD4+ receptor which can either be CCR5 or CXCR4. Then gp41 plays a role of mediating the fusion of the host and viral membrane. HIV genomic RNA and its associated proteins are deposited into the host cell where reverse transcriptase catalyzes the reverse transcription of the viral RNA to double-stranded viral DNA. The viral DNA translocates to the nucleus where it is integrated into the host cell genome by the help of the HIV enzyme integrase. The integrated proviral DNA in the nucleus is transcribed to form genomic viral RNA or mRNA which will be translated into viral proteins. Viral particles are formed by the assembly of the HIV proteins, enzymes and genomic RNA at the cell plasma membrane. Immature virus buds of at the cell membrane. After budding, the

HIV protease cleaves the Gag-Pol precursor proteins yielding a mature virion that is infectious and ready to infect new cells as shown in figure 1 below.

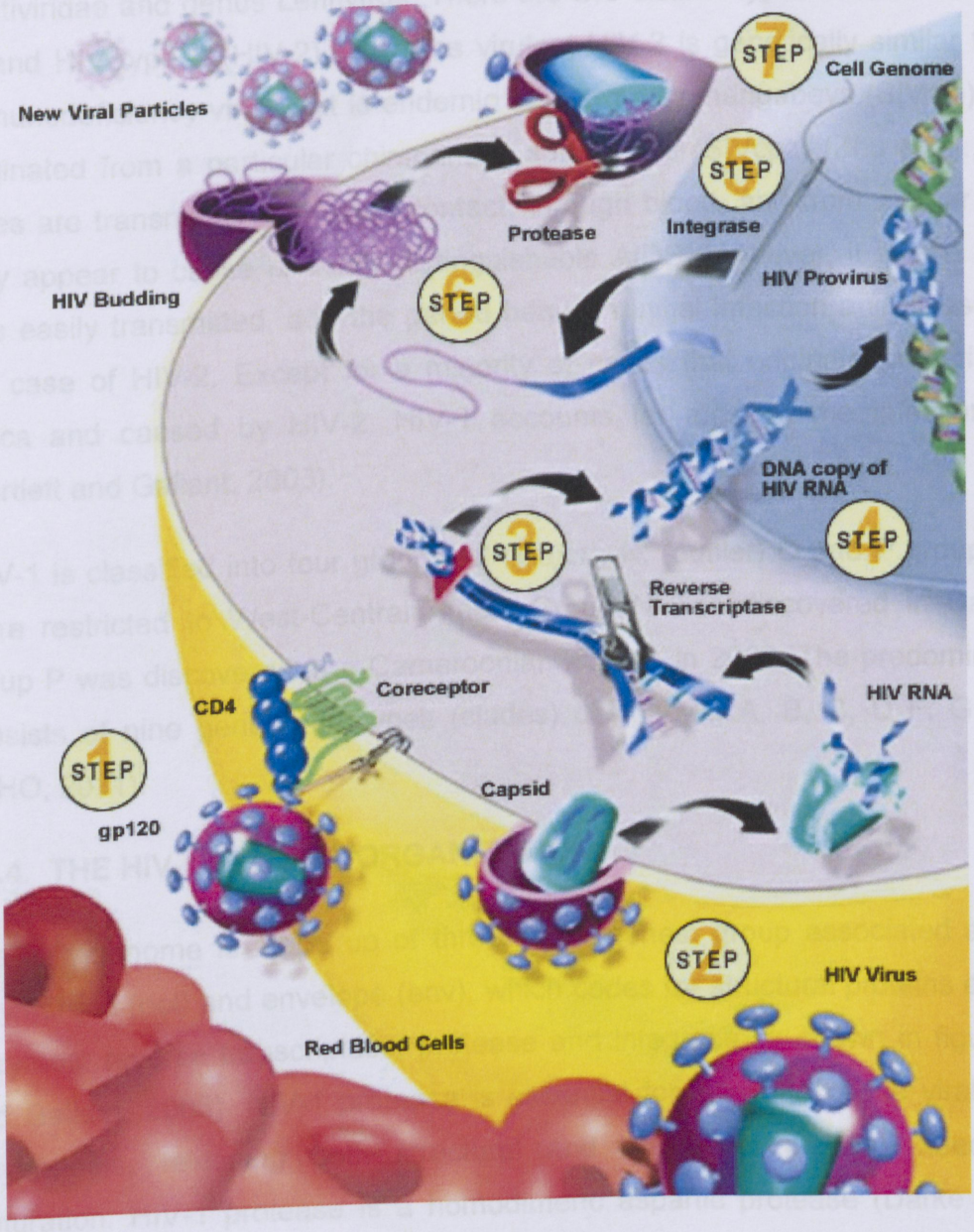

Figure 1: The HIV-1 replication cycle (www.mc.vanderbilt.edu) accessed on 03-06-13.

1.3. CLASSIFICATION AND GENETIC VARIABILITY OF HIV

HIV belongs to the family Retroviridae, subdivided into three subgroups: Spumavirus, Oncovirus and Lentivirus (Montagnier, 2002). HIV belongs to the subfamily of Lentiviridae and genus Lentivirus. There are two distinct types of HIV: HIV type 1 (HIV-1) and HIV type 2 (HIV-2). The less virulent HIV-2 is genetically similar to the simian immunodeficiency virus that is endemic among sooty mangabeys (SIVsm), while HIV-1 originated from a particular chimpanzee subspecies (SIVcpz) (Zhu et al., 1998). Both types are transmitted by sexual contact, through blood, and from mother to child, and they appear to cause clinically distinguishable AIDS. However, it seems that HIV-2 is less easily transmitted, and the period between initial infection and illness is longer in the case of HIV-2. Except for a minority of cases that originate primarily from West Africa and caused by HIV-2, HIV-1 accounts for most of the infections worldwide (Bartlett and Gallant, 2003).

HIV-1 is classified into four groups: M (Major), N, (outlier) O and P group. Group O is more restricted to West-Central Africa. Group N was discovered in Cameroon and group P was discovered in a Cameroonian woman in 2009. The predominant group M consists of nine genetic subtypes (clades) designated A, B, C, D F, G, H, J and K (WHO, 2011).

1.4. THE HIV-1 GENOME ORGANIZATION

The HIV genome is made up of three major genes: group associated antigen (gag), polymerase (pol) and envelope (env), which codes for structural proteins and three viral enzymes; reverse transcriptase, protease and integrase as shown in figure 2 (Bartlett and Gallant, 2003). HIV-1 protease is essential for cleavage of the viral gag and pol polyproteins, releasing both structural and enzymatic proteins needed for viral maturation. HIV-1 protease is a homodimeric aspartic protease (Darke et al., 1998). Integrase is a 32kDa protein with three distinct regions: the N-terminus, the core and the C-terminus. Integrase catalyzes the integration of cDNA copy of the genome into the host chromosome. Integrase has 3 enzymatic functions: (1) 3' end processing, where it

catalyzes the removal of two nucleotides from each 3' end of the viral DNA. (2) Strand transfer, IN catalyzes a concerted cleavage-ligation reaction resulting in viral DNA that is covalently attached to target DNA. (3) Disintegration is the process whereby IN can resolve a partially integrated piece of DNA into two separate entities (Bushman et al., 1993).

Rev is a 19kDa phosphoprotein located in the nucleus. Rev protein mediates the transition from early to late transcripts by allowing singly spliced or unspliced viral mRNA to accumulate in the cytoplasm (Emerman et al., 1989). Rev shuttling between the nucleus and cytoplasm is mediated by the nuclear localizing signal (NLS) and nuclear export signal (NES) thereby enabling the export of unspliced mRNA into the cytoplasm (Meyer and Malim, 1994). Nuclear import of Rev is initiated when its NLS binds to importin-β, which directs the complex to the nucleus where it combines with Ran-GDP in the cytoplasm. After translocation of Rev-importin-β complex into the nucleus, Ran-GDP is converted into Ran-GTP which causes Rev to dissociate from importin-β. In the nucleus, Rev protein binds to RRE and transport the importin-β, then returns it to the cytoplasm to be imported again (Suhasini and Reddy, 2009).

Tat protein is a HIV-1 transcriptional trans-activator and is essential for viral transcription. It binds to the transactivation response element (TAR), and is consequently involved in both transcriptional initiation and elongation.

Nef (Negative regulatory factor) forces the infected cell to stop making several proteins that are important in cell defense. Nef is important in the progression of HIV infection to AIDS (Geyer et al., 1999).

Vpr is an accessory HIV-1 viral protein which plays a role in the HIV-1 pathogenesis. Its deletion reduces the virulence of HIV-1 dramatically. Vpr is a 14kDa protein which is versatile and it composes of 96 amino acids (Muller et al., 2000). Vpr is incorporated into HIV-1 virions through direct interaction with the p6 domain of the Gag protein (Bachand et al., 1999). The functions of Vpr include modulation of transcription of the virus genome, induction of apoptosis, disruption of cell-cycle control, induction of

defects in mitosis, nuclear transport of the HIV-1 pre-integration complex (PIC), facilitation of reverse transcription, suppression of immune activation and reduction of the HIV mutation rate.

Vif (Viral infectivity factor) attacks one of the cell's defense proteins which force the cell to destroy (Stanley et al., 2008).

Vpu (Viral protein u) helps the virus to escape the cell during budding by weakening the interaction of the new envelope proteins with cell receptors (Willbold et al., 1997).

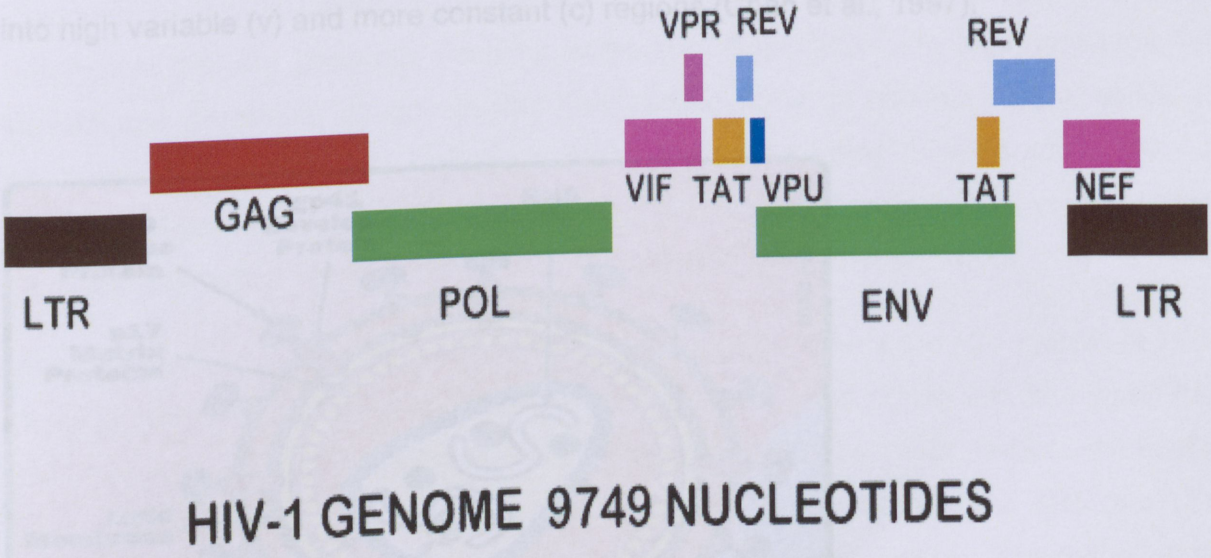

Figure 2: The structure of HIV-1 genome organization http://pathmicro.med.sc.edu (accessed on the 15-06-2013).

1.5. THE STRUCTURE OF HIV

The structure of HIV follows the typical pattern of the retrovirus family, consisting of a single-stranded, positive-sense ribonucleic acid (RNA) genome of about 9.7 kilo bases. HIV RNA consists of two strands and each has a copy of the virus's nine genes. The RNA is surrounded by a cone-shaped capsid which consists of approximately 2000 copies of the p24 viral protein as shown in figure 3. The capsid is covered by the viral envelope, which composes of a lipid bilayer membrane, formed from the cellular membrane of the host cell during budding of the newly formed virus particle. Envelope subunit consists of two non-covalently linked membrane proteins: glycoprotein 120 (gp120), the outer envelope protein, and glycoprotein 41(gp41), the transmembrane protein that anchors the glycoprotein complex to the surface of the virion. The envelope protein is the most differential component of HIV, although gp120 is structurally divided into high variable (v) and more constant (c) regions (Chan et al., 1997).

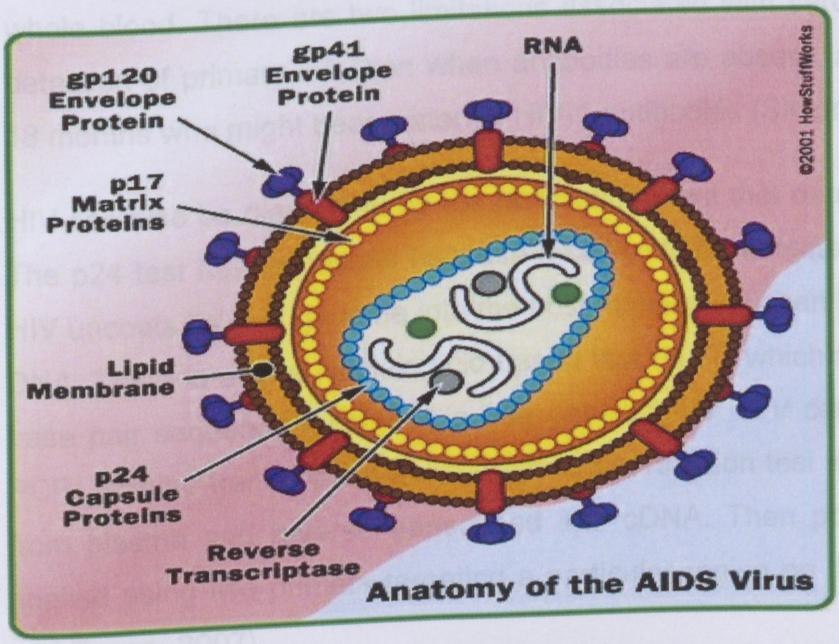

Figure 3: The structure of HIV (http://science.howstuffworks.com/life/aids2.htm accessed 15-08-2013).

1.6. TRANSMISSION AND PREVENTION OF HIV

HIV can be transmitted from (MTC) mother-to-child during pregnancy and also after birth during breastfeeding. HIV can get into the blood through cuts or sores on the skin. HIV is transmitted by sexual activities and from blood products and blood transfusion.

HIV MTCT can be reduced by caesarean section during delivery and also by taking one dose of nevirapine during pregnancy (Maguire et al., 1997). HIV can be prevented from MTCT by replacing breastfeeding with formula feeding (Dunn et al., 1992). HIV transmission can also be prevented by the use of condoms, wearing protective gloves if you are working in hospital or laboratories with HIV positive patients.

1.7. DIAGNOSIS OF HIV

Diagnosis of HIV is based on the detection of specific antibodies, antigens, or both. Serological test are generally used for screening. Rapid tests are important tools for surveillance, screening, diagnosis and they can be done on plasma, serum, saliva or whole blood. There are two limitations associated with serological tests which are the detection of primary infection when antibodies are absent, and in infants younger than 18 months who might bear maternal HIV-1 antibodies (Simon et al., 2006).

HIV can also be diagnosed by the p24 antigen test that detects the p24 protein of HIV. The p24 test has limitations because it can only be detected during the time when the HIV uncoats its viral genome into the host nucleus so it can be integrated into the host's DNA. There is also a Nucleic acid based test (NAT) which amplifies and detects a 124 base pair sequence located in a gag gene of HIV. HIV can also be detected by (RT-PCR) reverse transcription-polymerase chain reaction test where viral RNA is extracted from plasma and reverse transcribed into cDNA. Then polymerase chain reaction is applied using two primers targeting a particular region on the HIV genome (Armstrong and Taege, 2007).

1.8. TREATMENT OF HIV

Up to date, there are 31 approved antiretroviral drugs by the Food and Drug Administration (FDA) for the HIV infection treatment. These antiretrovirals (ARVs) suppress the virus even to undetectable levels but they do not completely eliminate it from the body, therefore, AIDS cannot be cured. HIV treatment is divided into five classes, the Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs), Integrase Inhibitors (IN) and Fusion Inhibitors (FIs).

Nucleoside Reverse Transcriptase Inhibitors include: Abacavir (ABC), Stavudine (d4T), Didanosine (ddI), Tenofovir (TDF), Emtricitabine (FTC), Zidovudine (AZT) and Lamivudine (3TC). Non-Nucleoside Reverse Transcriptase Inhibitors include: Delavirdine, Efavirenz (EFV), Etravirine, Nevirapine (NVP) and Rilpivirine. Protease Inhibitors blocks the HIV protease from cleaving the gag-pol polyproteins into functional proteins. They include, Atazanavir, Nelfinavir, Ritonavir, Darunavir, Saquinavir, Fosamprenavir, Tripanavir and Indinavir. Integrase Inhibitors which blocks the integration of HIV into the host nucleus include Raltegravir and Elvitegravir. CCR5 antagonist which blocks the HIV from attaching to the CCR5 of a CD4 cell for entry includes maraviroc (National Institute of Allergy and Infectious Diseases, April 2013).

Nucleoside Reverse Transcriptase Inhibitors prevents HIV from multiplying. Zidovudine alone or in combination with other ARVs is recommended for HIV-infected pregnant women to prevent HIV from spreading to the fetus. The baby also receives treatment for 6 weeks after birth. The first line regimen of antiretrovirals includes Tenofovir, Lamivudine, or Emtricitabine with Efavirenz or Nevirapine for pregnant women or women of child bearing age.

For TB co-infected patients, the preferred ARV is Efavirenz. The second line regimen for those who fail first line treatment includes tenofovir, Lamivudine or Emtricitabine with lopinavir/ritonavir.

1.9. HEPATITIS C VIRUS

Hepatitis C is an infectious disease that primarily affects the liver, caused by the hepatitis C virus (HCV) (Ryan and Ray, 2004). HCV was identified in 1988 through molecular biology techniques. The virus can be spread by blood-to-blood contact or may be sexually transmitted, although it is rare. The infection is often asymptomatic but it can be chronic and cause liver failure or other complications such as liver cancer or cirrhosis. Symptoms of acute hepatitis C infection include decreased appetite, fatigue, abdominal pain, jaundice, itching and flu-like symptoms.

Laboratory assays that are available for the diagnosis and management of HCV infection include: (i) serologic tests to detect HCV antibodies, (ii) molecular tests to detect and quantify HCV RNA, and (iii) genotyping techniques.

HCV has six genotypes numbered 1-6 and a large number of subtypes that have been described. Genotype 1 is the most prevalent genotype worldwide, with a higher prevalence of 1b in Europe and 1a in the US. Genotype 3a is highly prevalent in European intravenous drug users. Genotype 2 is found in clusters in the Mediterranean region, while 5 and 6 are rarely found (Antaki et al., 2010). It is estimated that approximately 130-210 million individuals worldwide are chronically infected with HCV which gives the prevalence of 3%. In Western Europe, HCV prevalence ranges from 0.4% to 3%. Egypt has the highest worldwide prevalence, with 9% countrywide and up to 50% in certain rural areas, due to specific modes of infection (Esteban et al., 2008).

Liver disease progression takes place over several decades, and is accelerated in the presence of cofactors such as alcohol consumption, diabetes mellitus, and older age of acquisition, HIV co-infection or co-infection with other hepatotropic viruses.

1.10. VIROLOGY OF HCV

HCV is a small (50nm in size), enveloped, single-stranded, positive sense RNA virus (Bendinelli et al., 2000). The hepatitis C virus belongs to the Flaviviridae family. It is the only member of the Hepacivirus genus. Hepatitis C virus is a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma worldwide (National Institute of Health Consensus Development Conference Statement, 2002).

1.11. STRUCTURE OF HCV

The HCV is spherical in shape; the size of the viral particle is between 36 and 62nm (Pozetto et al., 1996). The structure consists of a core of RNA, surrounded by an icosahedral protective shell of protein, and further encased in a fatty envelope of cellular origin. The viral nucleocapsid, consisting of core protein and viral genomic RNA, is enveloped by a lipid bilayer containing two glycoproteins (E1 and E2) as shown in figure 4 (Forns and Bukh, 1999) (Figure 4).

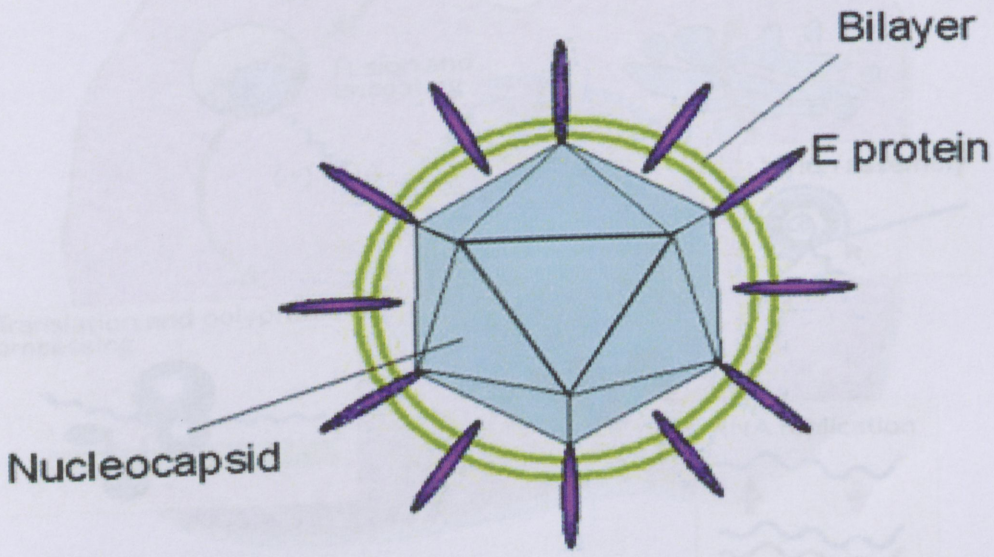

Figure 4: The structure of HCV virion (www.usg.edu/ehs/training/pathogens/images/p10.gif accessed on the 11-01-2012).

1.12. THE HCV LIFE CYCLE

The HCV binds to one or more cellular receptors that are organized as a receptor complex and appear to trigger receptor-mediated endocytosis. The virion envelope will fuse with cellular membrane to deliver the nucleocapsid to the cytoplasm where translation of the viral genome takes place leading to the production of a polyprotein (Figure 5). The polyprotein will then be cleaved into structural and nonstructural proteins by both cellular and viral proteases. After replication, progeny virions are assembled from cytoplasmic vesicles formed by budding through intracellular membranes. In the last step of the HCV life cycle, mature virions are released into the extracellular milieu by exocytosis. The process is shown in figure 5.

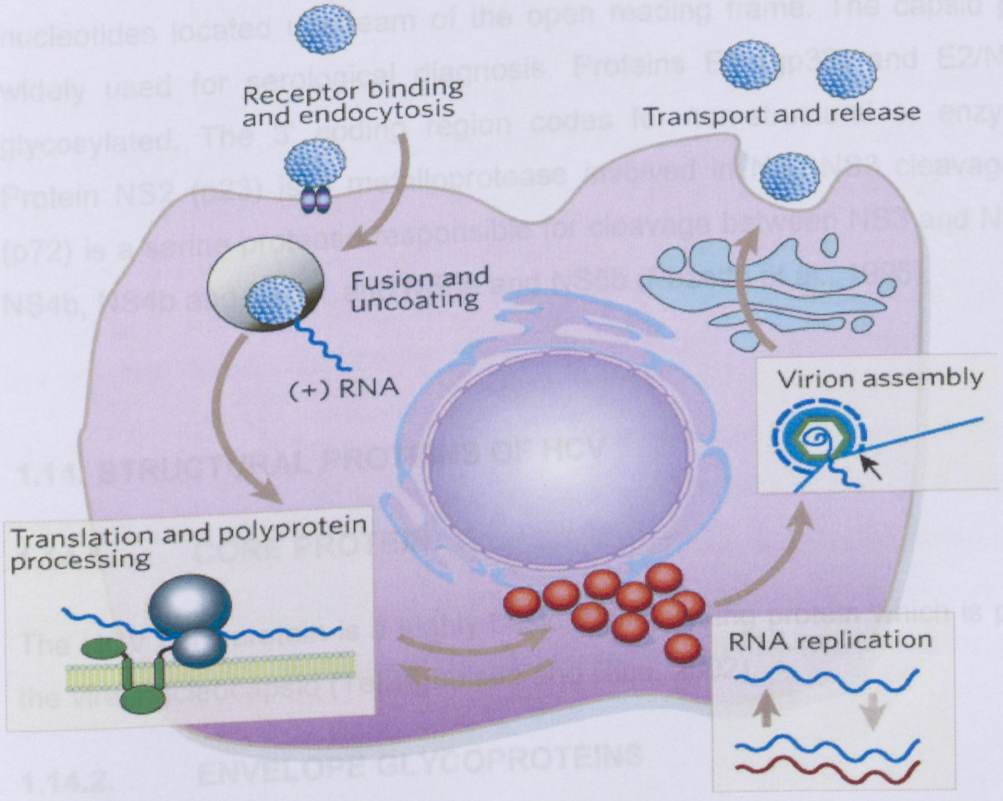

Figure 5: The structure of the HCV life cycle (www.nature.com/.../images/nature04077-f1.2.jpg accessed on the 11-01-2012).

1.13. GENOME ORGANIZATION OF HCV

HCV is a linear RNA virus with a positive-sense single stranded genome of approximately 9,400 nucleotides, it consists of two non-coding regions in 5' and 3' flanking a long reading frame coding for a polyprotein of approximately 3,000 amino acids. The 5' non-coding region is the most conserved part of the genome. The 5' coding region corresponds to the capsid (C) and envelope (E1 and E2) proteins (structural region) (Pozzetto et al., 1996). The 5' UTR is considered important in initiating translation of the viral genome, while conserved elements within the 3' UTR are essential for RNA synthesis and genome packaging (Forns and Bukh, 1999).

The HCV 5' UTR is the most conserved region of the genome and it contains 341 nucleotides located upstream of the open reading frame. The capsid protein (p22) is widely used for serological diagnosis. Proteins E1 (gp33) and E2/NS1 (gp72) are glycosylated. The 3' coding region codes for non-structural or enzymatic proteins. Protein NS2 (p23) is a metalloprotease involved in NS2-NS3 cleavage. Protein NS3 (p72) is a serine-protease responsible for cleavage between NS3 and NS4a, NS4a and NS4b, NS4b and NS5a, and NS5a and NS5b (Pozetto et al., 1996).

1.14. STRUCTURAL PROTEINS OF HCV

1.14.1. CORE PROTEIN

The HCV core protein is a highly basic, RNA-binding protein which is presumed forms the viral nucleocapsid (Tellinghuisen and Rice, 2002).

1.14.2. ENVELOPE GLYCOPROTEINS

The envelope glycoproteins E1 and E2 are type I transmembrane proteins with C-terminal hydrophobic anchors. E1 and E2 form non-covalent heterodimers (Wakita et al., 2005).

1.14.3. P7 protein

P7 is a 63-amino acid polypeptide located at the junction between the structural and non-structural region. P7 is composed of two transmembrane domains (Pavlovic et al., 2003).

1.15. NON-STRUCTURAL PROTEINS

1.15.1. NS2-3 AUTOPROTEASE

The NS2/3 junction is cleaved by a remarkable autoprotease consisting of NS2 and the N-terminal third of NS3.

1.15.2. NS3-4A

NS3 is a serine protease that belongs to the trypsin/chymotrypsin protease super family. This enzyme consists of two β-barrel domains that are flanked by two short alpha helices (Raney et al., 2010). It is a multifunctional protein because it harbors a serine protease located in the N-terminal one-third that is responsible for the downstream cleavage in the non-structural region and NTPase/RNA helicase domain in the C-terminal two thirds.

NS4A is a 54 amino acid polypeptide which is required as a co-factor for the NS3 serine protease. The NS4A is an integral component of the enzyme core (Kim et al., 1996). The enzymatic activity of the NS3 NTPase/helicase activity is indispensable for RNA replication. Putative functions during replication could be to unwind replicative double strand RNA intermediates, to remove RNA secondary structures or to separate the genome from nucleic acid binding proteins (Serebrov and Pyle, 2004).

1.15.3. NS4B

NS4B is a 27kDa integral membrane protein that localizes to an ER-derived membranous compartment. The expression of NS4B induces a specific membrane

alteration, designated as membranous web that serves as a scaffold for the formation of the viral replication complex (Egger et al., 2002).

1.15.4. NS5A

NS5A is a phosphorylated zinc metalloprotein and it plays an important role in the regulation of viral replication. The membrane association of NS5A is mediated by a unique amphipathic alpha-helix which is localized at the N terminus.

1.15.5. NS5B

NS5B RNA-dependent RNA polymerase is the key enzyme of the replicase that promotes synthesis of new RNA genomes. NS5B is a tail-anchored protein, characterized by a transmembrane domain at the C-terminus of the protein responsible for post translational membrane targeting.

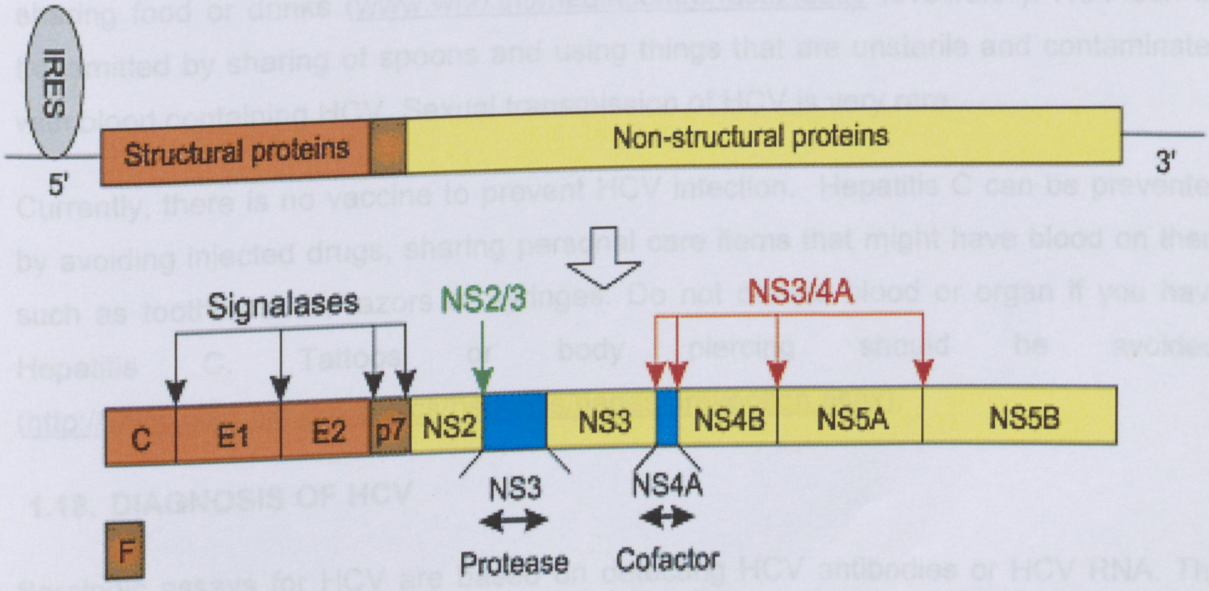

Figure 6: The structure of HCV genome organization (http://journal.prous.com accessed on the 11-01-2012)

1.16. HCV PREVALENCE

It is estimated that 3-4 million people are infected with HCV each year and more than 350 000 people die from HCV-related liver diseases. Countries with high rates of chronic infection are Egypt (22%), Pakistan (4,8%) and China (3,2%). Chronic HCV affects 170 million individuals worldwide, with 10% to 15% of cases leading to cirrhosis and liver cancer (WHO, 2011).

1.17. TRANSMISSION AND PREVENTION OF HCV

Hepatitis C transmission occurs through receipt of contaminated blood transfusions, blood products and organ transplants. It can also be transmitted through injections given with contaminated syringes and needle-stick injuries in health care settings. HCV can be also transmitted by being born to a hepatitis C-infected mother but it cannot be spread through breast milk, water, food or casual contact such as hugging, kissing and sharing food or drinks (www.who.int/mediacentre/factsheets/ fs164/en/). HCV can be transmitted by sharing of spoons and using things that are unsterile and contaminated with blood containing HCV. Sexual transmission of HCV is very rare.

Currently, there is no vaccine to prevent HCV infection. Hepatitis C can be prevented by avoiding injected drugs, sharing personal care items that might have blood on them such as toothbrushes, razors or syringes. Do not donate blood or organ if you have Hepatitis C. Tattoos or body piercing should be avoided. (http://www.niad.nih.gov/topics/hepatitis/pages/prevention.aspx).

1.18. DIAGNOSIS OF HCV

Serologic assays for HCV are based on detecting HCV antibodies or HCV RNA. The most commonly used serologic test is the enzyme-linked immunosorbent assay with a sensitivity and specificity of 95%. Confirmation of ongoing infection therefore requires the detection of HCV RNA by polymerase chain reaction (PCR) using a qualitative or quantitative assay. These assays may detect a viral count as low as 9.6IU/L. Positive

results by enzymeimmuno assay (EIA) must be confirmed by the recombinant immunoblot assay (RIBA).

1.19. TREATMENT FOR HCV

The Food and Drug Administration has approved two antiviral drugs for the treatment of chronic hepatitis C: Alpha Interferon or pegylated interferon and ribavirin. Interferon is given alone or in combination with ribavirin for 12 months (www.cdc.gov/idu). Ribavirin is a pro-drug which interferes with the RNA metabolism which is required for viral replication (Feld and Hoofnagle, 2005).

There are two new therapeutic antiviral drugs that have been recently licensed in some countries for the people who are failing treatment. Telaprevir and boceprevir are the new drugs for Hepatitis C treatment. Telaprevir is the target molecule of the NS3-4A serine protease (Clercq, 2007). Telaprevir and boceprevir are HIV protease inhibitors which were approved in 2011 to be used in combination with PegIFN and Ribavirin to treat Hepatitis C in treatment-naïve and experienced HCV genotype-1 infected patients because they were found to be more effective when used in combination (Yee et al., 2012).

1.20. HUMAN PAPILLOMAVIRUS

Human papillomavirus (HPV) is the most common sexually transmitted infection today. HPV infects squamous epithelial cells in the cervix, glands of the penis. Human papillomaviruses infect only humans. There are about 100 types of HPV that affect different parts of the body. There are more than 40 HPV types that can infect the genital areas of males and females. These HPV types can also infect the mouth and throat. HPVs are highly epitheliotropic viruses, of which more than 180 genotypes have been identified and are divided into cutaneous or mucosal categories based on their tissue tropism (de Villiers et al., 2004). Several genotypes are defined as high-risk genotypes and are associated with invasive diseases, while the other genotypes are considered low-risk genotypes since they are not associated with the development of cervical carcinoma (Munoz et al., 2006).

Genitally transmitted HPV types are contained within supergroup A also known as Alpha papillomaviruses (de Villiers et al., 2004). Viruses from the supergroup A, such as HPV 6 and 11 are major sexually transmitted pathogens. There are high-risk viruses from supergroup A, such as HPV-16 and HPV-18 which cause mucosal lesions that can progress in some individuals to high-grade neoplasia and cancer (Bosch et al., 2002). HPV-2 and closely related supergroup A papillomaviruses are the primary cause of common warts.

The second major group of human papillomaviruses is contained within supergroup B. Viruses from the subgroup B1 such as HPV5 also known as Beta papillomaviruses causes latent infections in the general population, but can become a problem in immune-suppressed and individual who have an inherited defect which renders them susceptible to infection by papillomaviruses. Viruses from the B2 subgroup such as HPV-4 also known as Gamma papillomaviruses causes cutaneous warts in the general population. The last HPV group falls within supergroup E also classified as Mu and Nu-papillomaviruses (de Villiers et al., 2004).

1.20.1. VIROLOGY OF HPV

Human papillomavirus (HPV) is a small deoxyribonucleic acid virus of approximately 7,900 base pairs which causes cervical cancer in females. Papillomaviruses are small non-enveloped icosahedral viruses which are 52-55nm in diameter. The human papillomaviruses belongs to the Papillomaviridae family.

1.20.2. HPV GENOME

HPV genome comprises of early and late genes that encode early proteins E1-E7 and late proteins L1-L2 as shown in figure 7. It contains eight genes with multiple promoters and a number of variants that are expressed either early or late in the HPV lifecycle. The early genes encode nonstructural proteins that participate in DNA replication, transcriptional regulation, cell transformation and viral assembly and release whereas the late genes L1 and L2 encode viral capsid proteins (Bernard, 2002).

E1 and E2 are both DNA binding proteins. E1 is a DNA helicase/ATPase involved in viral genome replication while E2, is a transcription factor that binds to four sites within the viral noncoding region and therefore, loss of E2 during integration of viral DNA into the host genome is the first stage in transformation (Talora et al., 2002).

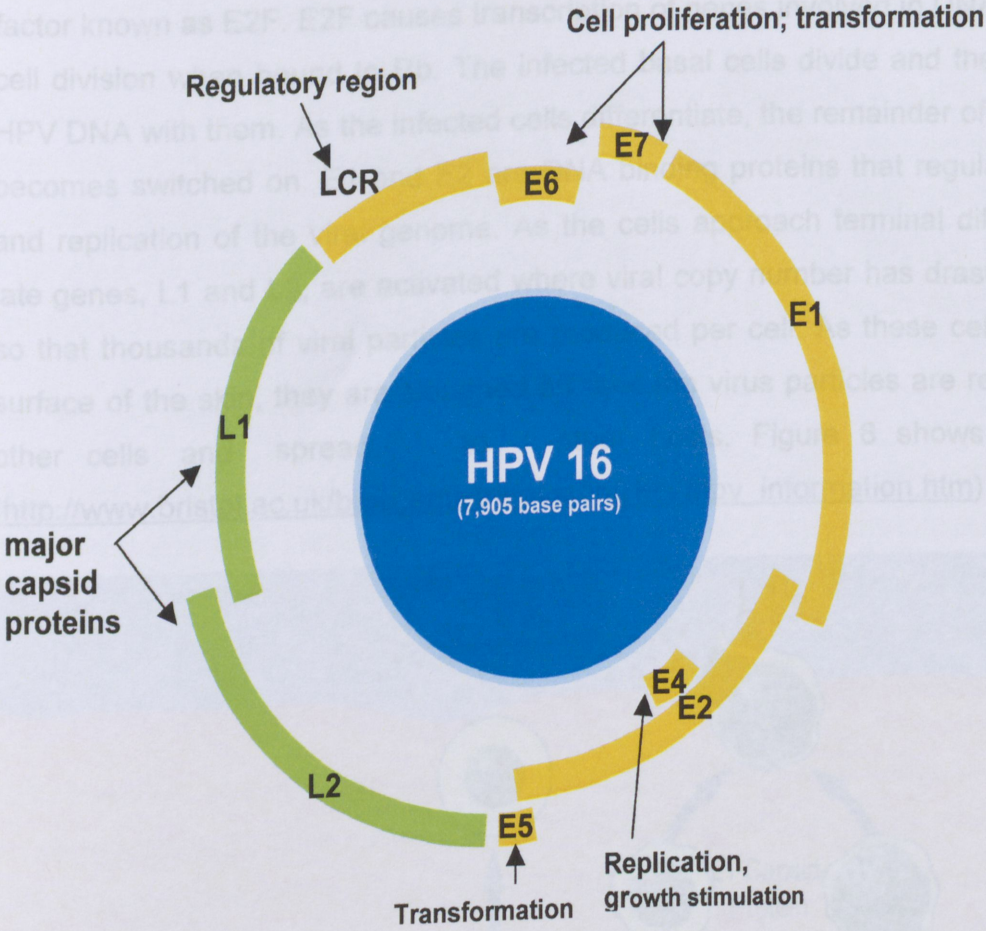

Figure 7: General organization of the HPV genome (Villa, 2006)

1.20.3. HPV LIFE CYCLE

Human papillomavirus infects basal epithelial cells via minor abrasions on the skin. It enters a cell, uncoats and delivers its double stranded DNA to the cell's nucleus. E6 and E7 are the first genes to be expressed and they are involved in cell transformation. E6 bind to and inhibits p53, which is active to repress the cell cycle in the event of DNA damage and it also triggers apoptosis. E6 will then activate cellular telomerase, the enzyme that synthesizes the telomere repeat sequences at the ends of eukaryotic chromosomes which allows the cells to replicate indefinitely. E7 promotes cell division by binding to Rb, a tumor suppressor protein that binds to and inactivates a transcription

factor known as E2F. E2F causes transcription of genes involved in DNA replication and cell division when bound to Rb. The infected basal cells divide and their progeny take HPV DNA with them. As the infected cells differentiate, the remainder of the early genes becomes switched on. E1 and E2 are DNA binding proteins that regulate transcription and replication of the viral genome. As the cells approach terminal differentiation, the late genes, L1 and L2, are activated where viral copy number has drastically increased so that thousands of viral particles are produced per cell. As these cells approach the surface of the skin, they are sloughed off, and the virus particles are released to infect other cells and spread to other hosts. Figure 8 shows the life cycle. (http://www.bristol.ac.uk/biochemistry/gaston/HPV/hpv_information.htm)

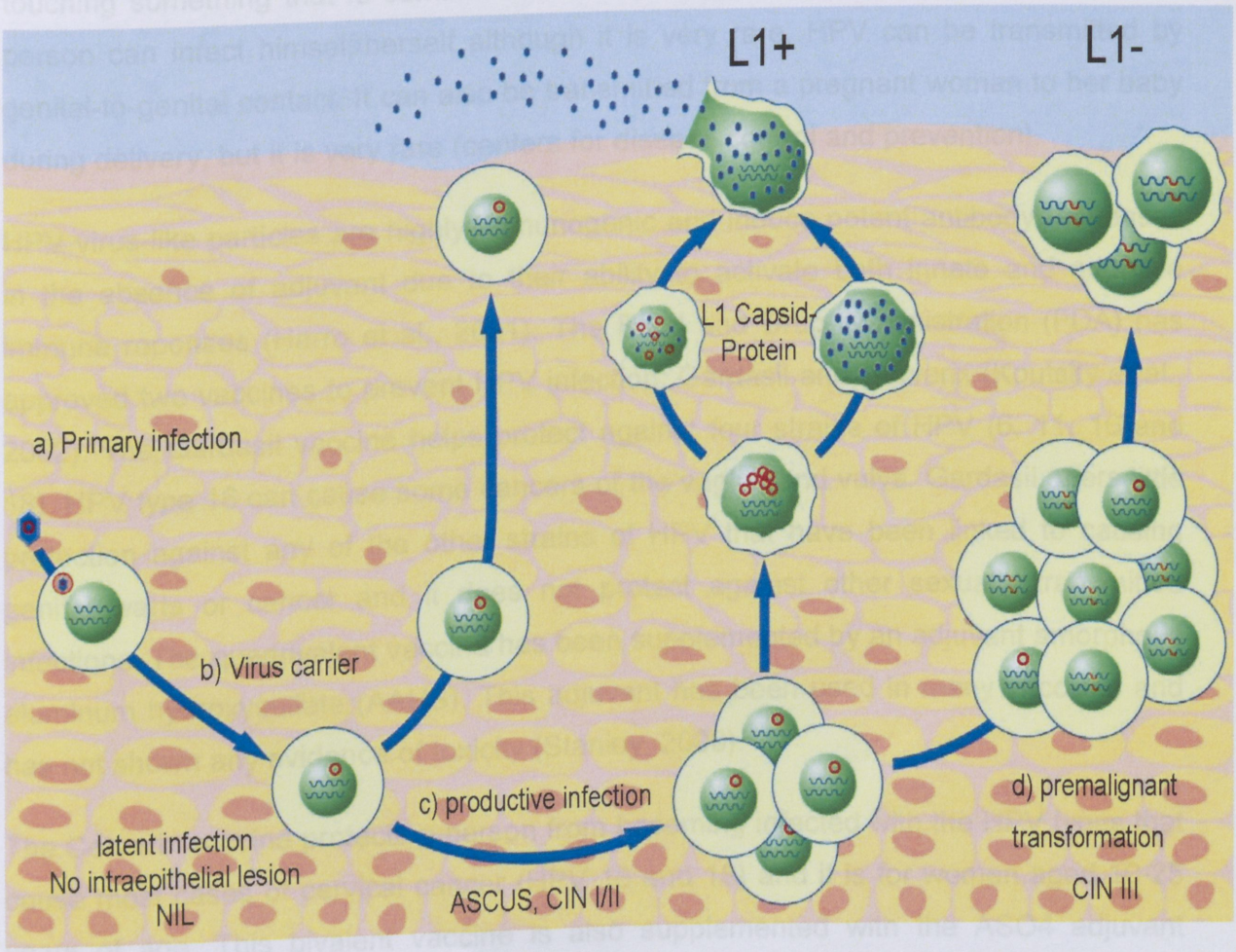

Figure 8: The structure of HPV life cycle (Hilfrich, 2013).

1.20.4. THE PREVALENCE OF HPV

Approximately 6000 new cervical cancer cases are diagnosed annually in South Africa. Accurate contemporary data on cervical incidence have not been available since 1999 Cancer Registry was published (Mqoqi et al., 2004). The world Health Organization estimates the age-standardized incidence rate for SA to be 26.6 per 100 000 women (WHO/ICO Information Centre on HPV and Cervical Cancer, South Africa 2010).

1.20.5. TRANSMISSION AND PREVENTION OF HPV

HPV can be transmitted through direct skin-to-skin contact. It can also be spread by touching something that is contaminated with the virus and then touching the skin. A person can infect himself/herself although it is very rare. HPV can be transmitted by genital-to-genital contact. It can also be transmitted from a pregnant woman to her baby during delivery, but it is very rare (centers for disease control and prevention).

HPV virus-like particles are highly immunogenic and induce potent antibody responses in the absence of adjuvant due to their ability to activate both innate and adaptive immune reponses (Harro et al., 2001). The Food and Drug Administration (FDA) has approved two vaccines to prevent HPV infection: Gardasil and Cervarix (Koutsky et al., 2002). The Gardasil vaccine helps protect against four strains of HPV (6, 11, 16 and 18). HPV type 16 can cause some cancers of the vagina and vulva. Gardasil offers little protection against any of the other strains of HPV that have been linked to causing genital warts or cancer and it does not protect against other sexually transmitted infections. The quadrivalent vaccine has been supplemented by an adjuvant amorphous aluminum hydroxysulfate (AAHS). This adjuvant has been used in many vaccines and has not shown any evidence of toxicity (Stanley, 2008).

The Cervarix vaccine protects a person from becoming infected with the HPV types that cause most cases of cervical cancer (HPV 16 and 18) and it is for women aged 10-25 years of age. This bivalent vaccine is also supplemented with the ASO4 adjuvant

system which comprises of aluminum hydroxide and monophosphorylnlipid A (MPL) which is a modified endotoxin and agonist of TLR4 (Stanley, 2008).

1.20.6. DIAGNOSIS OF HPV

HPV can be diagnosed by Pap test which involves the examination of cervical cells to look if there are changes in the cells. Another method for HPV diagnoses is by Colposcopy, which involves the use of an instrument called colposcope to shine a light and magnifies the view of the cervix and a vinegar solution is placed on the cervix inorder to turn the abnormal cells white. It can also be diagnosed by commercial ELISA kits which detects the presence of antibodies in a sample. The last method is by detecting the HPV DNA by polymerase chain reaction.

1.20.7. TREATMENT OF HPV

There is no cure for HPV but warts can be removed from the genitals to relieve symptoms. Sometimes treatment is not needed because many HPV infections resolve by themselves. Several methods are used to remove/treat warts including: (1) Cryosurgery, where warts are frozen with liquid nitrogen so they can be removed easily. (2) Loop electrosurgical excision procedure (LEEP), which uses a special wire loop to remove abnormal cells. (3) Electrocautery, which uses electric current to burn off the warts. (4) Laser therapy uses an intense light to destroy the warts and any abnormal cells.

1.21. CYTOMEGALOVIRUS

Cytomegalovirus (CMV) is a β-herpesvirus and it is known as human herpesvirus 5 (HHV-5). It is a member of the human herpseviridae family. Its classification is based on its tendency to infect mononuclear cells and lympocytes (Ridley, 1997). Cytomegalovirus is a type of herpes virus that often causes asymptomatic infection in otherwise healthy persons. Cytomegalovirus remains the leading cause of congenital viral infection and a significant cause of transfusion-acquired infections in the immunocompromised. CMV is the leading cause of congenital infection in the US. Although most CMV infections are asymptomatic, certain patient groups are at risk of developing serious illness and long term sequelae from CMV.

Human cytomegalovirus has three genes which allow it to evade human immunity and are important for the development of vaccine. The first gene is the US3 gene which gives off products that function to prevent the major histocompatibility complex molecules from moving to the membrane. The second one is the US6 gene whose products inhibit peptide translocation of proteins involved with antigen presentation. The last one is the US11 gene whose products makes the class I heavy chain of the antigen nonfunctional by moving it from the endoplasmic reticulum to the cytosol (Loenena et al., 2001).

1.21.1. STRUCTURE OF CYTOMEGALOVIRUS

Cytomegalovirus has an icosahedral capsid that surrounds and protects the genetic material. This icosahedral shape is composed of 20 equivalent triangle faces, shaped into a sphere. The capsid is made out of 162 hexagonal protein capsomeres, and is surrounded by a lipid bilayer outer envelope. On the envelope, there are glycoproteins. In between the outer envelope and the capsid, is the viral tegument, which is a proteinaceous layer which contains proteins involved in immunity (Figure 9).

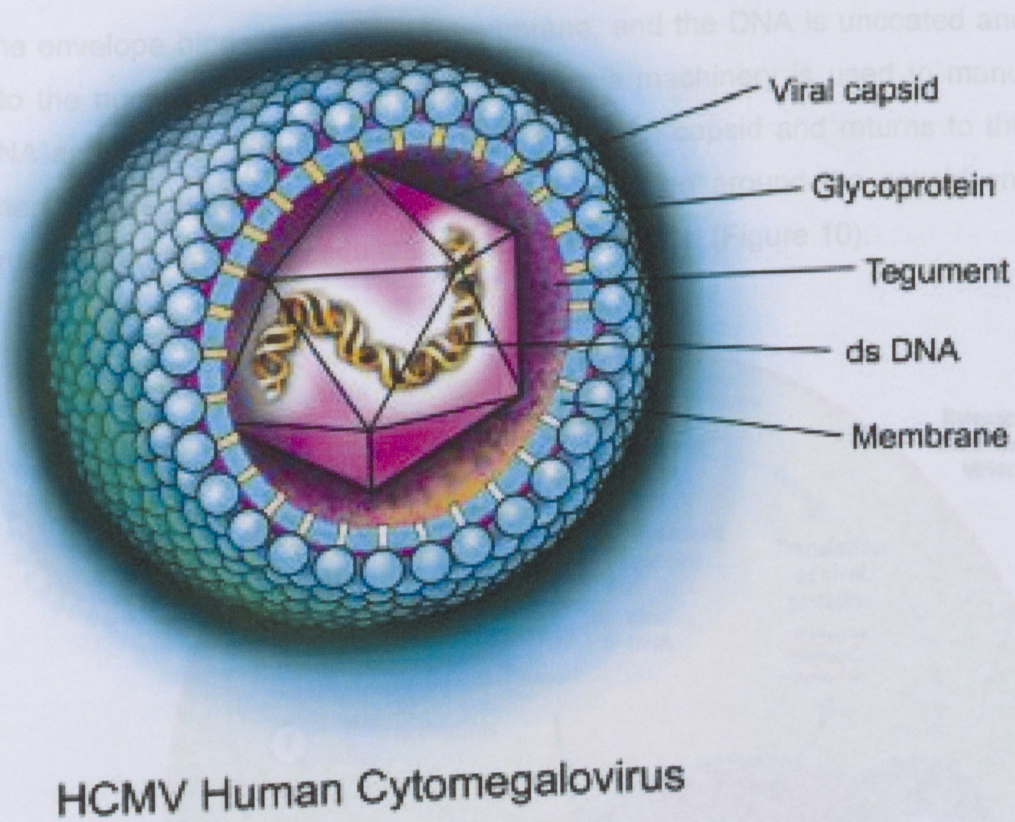

Figure 9: The structure of cytomegalovirus.

(http://cytomegalovirusproject.wikispaces.com accessed on the 18-09-2013).

1.21.2. CMV GENOME

The genome of human CMV consists of a linear molecule of double-stranded DNA containing 229,354 base pairs (Chee et al., 1990). The genome is divided into two regions: the unique short region (Us) which is 35,418 base pairs in length and bounded by two 2,524 base pair repeat sequences designated IRs and TRs and the unique long region (UL) which is 169,972 base pair in length and is flanked by two repeats of 11,247 base pair IRL and TRL (Weston and Barrell, 1986).

1.21.3. LIFE CYCLE OF CYTOMEGALOVIRUS

The envelope binds with the cell membrane, and the DNA is uncoated and transferred into the nucleus, where cell protein synthesis machinery is used to manufacture new DNA and capsid. The DNA is packaged into the capsid and returns to the cytoplasm, where the tegument and envelope are assembled around the capsid and the whole virus transported to the cellular surface and released (Figure 10).

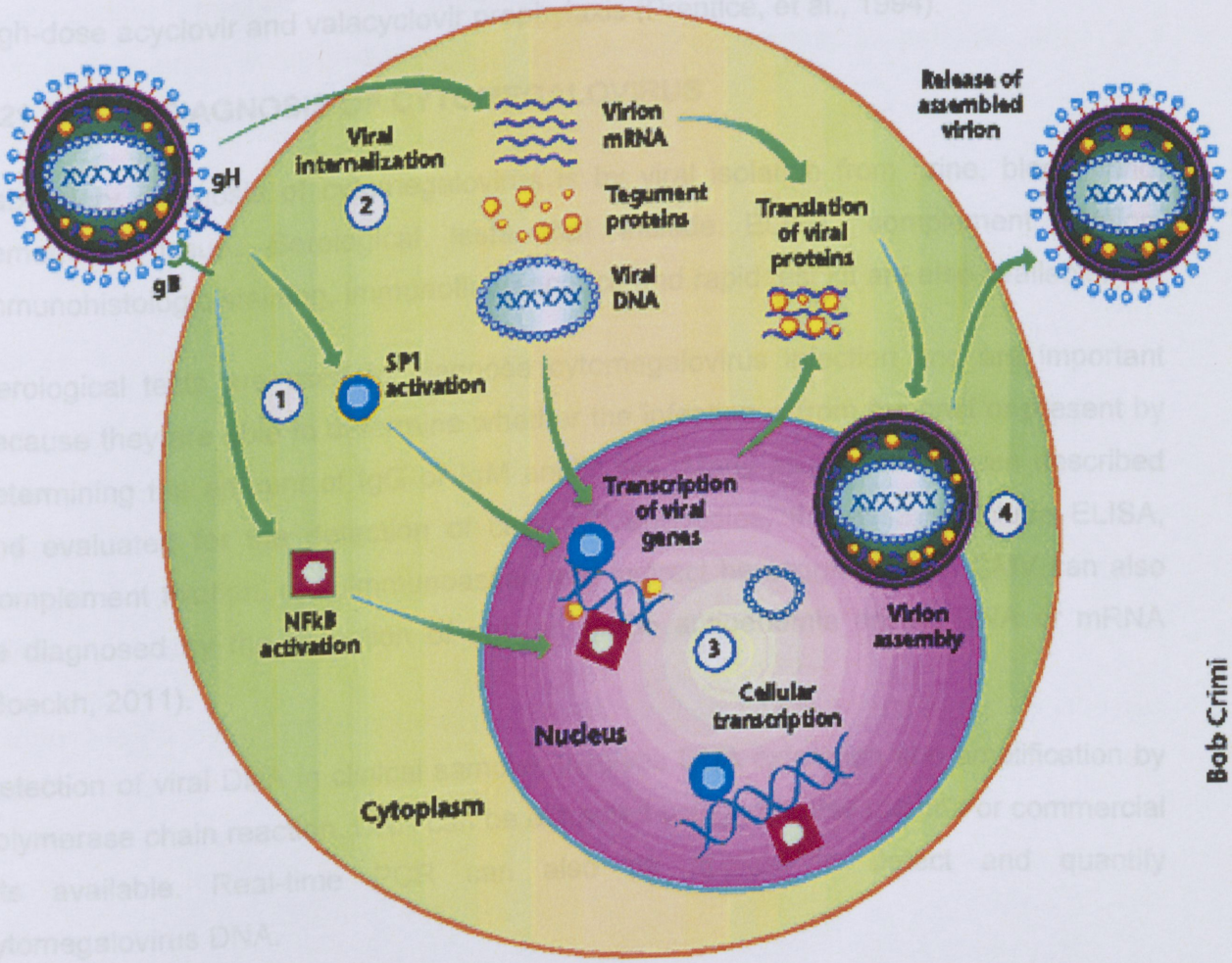

Figure 10: The human cytomegalovirus lytic life cycle (Huang and Johnson, 2000).

1.21.4. TRANSMISSION AND PREVENTION OF CYTOMEGALOVIRUS

The virus is passed from infected people to others through direct contact with body fluids, such as urine, saliva or breastmilk. It is also sexually transmitted. CMV can be spread through transplanted organs and blood transfusions. Cytomegalovirus infections can be further prevented at the blood bank by reducing and filtering leukocyte and following quality standards. Blood from cmv-seronegative donors can be used (Pollack, et al., 2011). Cytomegalovirus infection can also be reduced by the administration of high-dose acyclovir and valacyclovir prophylaxis (Prentice, et al., 1994).

1.21.5. DIAGNOSIS OF CYTOMEGALOVIRUS

Laboratory diagnosis of cytomegalovirus is by viral isolation from urine, blood, lung, semen or tissue. Serological tests that include ELISA, complement fixation, immunohistologic staining, immunofluorescence and rapid test kit are also available.

Serological tests are used to diagnose cytomegalovirus infection and are important because they are able to determine whether the infection is from the past or present by determining the amount of IgG or IgM antibodies. Many assays have been described and evaluated for the detection of cmv IgG antibodies, the assays include ELISA, Complement fixation, radioimmunoassay and indirect hemagglutination. CMV can also be diagnosed by the detection of antigen (pp65 antigenemia assay, DNA or mRNA (Boeckh, 2011).

Detection of viral DNA in clinical samples involves DNA extraction and amplification by polymerase chain reaction. DNA can be extracted using in-house method or commercial kits available. Real-time PCR can also be applied to detect and quantify cytomegalovirus DNA.

1.21.6. TREATMENT OF CYTOMEGALOVIRUS

Cytomegalovirus can be treated by using antiviral medicines such as valaciclovir, valganciclovir, foscarnet and cidoforvir. Valaciclovir is an antiviral drug that slows the growth and spread of the herpes virus. This drug is used to treat infections in adults and children.

Valganciclovir is an orally administered prodrug that is hydrolyzed to ganciclovir. Valganciclovir treats CMV infection of the eye in patients with acquired immunodeficiency syndrome and it prevents CMV infection in patients who have received an organ transplant.

Ganciclovir is an anti-viral drug which is used to prevent disease caused by cytomegalovirus in people who have received organ or bone marrow transplant. Ganciclovir is also used to treat people with weakened immune systems who have CMV retinitis.

Foscarnet is an antiviral medication used to treat herpes viruses, including drug resistant cytomegalovirus. Foscarnet is particularly used to treat CMV retinitis. This antiviral can be used to treat highly treatment experienced patients with HIV as part of salvage therapy. Foscarnet is used for the treatment of ganciclovir-resistant CMV infections in patients with AIDS or in transplant recipients.

Cidofovir is an injectable antiviral which is used with probenecid to treat certain viral eye infection due to CMV in people with AIDS. Cidofovir lowers the risk of blindness and other vision problems.

2. RATIONALE OF THE STUDY

Human immunodeficiency virus (HIV) is the causative agent of acquired immunodeficiency syndrome (AIDS). Majority of people infected with HIV, are also co-infected with other diseases that are caused by opportunistic pathogens such as Human papillomavirus, Cytomegalovirus and Hepatitis C virus. These diseases are asymptomatic, self-limiting and can cause chronic diseases later in human life. Although these diseases are expected in HIV infected individuals because their immune systems are weak, they are also present in HIV negative individuals and most of them are not aware and they become carriers and transmit it to other people. In South Africa, there is a high prevalence of cervical human papilloma virus (HPV) in women of child bearing age, especially oncogenic HPV type infection and there is currently limited knowledge about the individual HPV types associated with cervical diseases. Sexually active women have a lifetime risk of up to 80% to be infected with one or more HPV types.

Enzyme-linked immunosorbent assay (ELISA) is a diagnostic method used in detecting the presence of an antibody or an antigen in a particular sample. This immunological technique is a plate-based assay which was conceptualized and developed by Peter Perlmann, principal investigator, and Eva Engvall at Stockholm University in 1971 in Sweden. ELISA is a very sensitive and specific method and is important because it can provide information on whether the disease is primary or of secondary infection. Polymerase chain reaction is a molecular method that detects the presence of DNA in very low concentration of sample and also amplifies a target gene. It is also a very sensitive and specific method.

Many studies have been done in Africa generally, but not enough studies have been done in South Africa on HPV, CMV, and HCV, especially in the Limpopo province. So this study was conducted in order to determine the prevalence of cytomegalovirus, human papillomavirus and hepatitis C in HIV positive and negative groups. The prevalence of IgG antibodies against CMV, HCV and HPV were determined by the ELISA method because it is more sensitive than other serological methods and the

circulating HPV genotypes were determined by the Linear Array Genotyping Test kit from Roche which determines 37 high and low risk HPV types which is so far the only kit that detects many different types.

3. GENERAL OBJECTIVE OF THE STUDY

The general objective of the study was to determine the prevalence of CMV,HPV and HCV, and also to determine the genotypes of HPV in Northern South Africa.

3.1. SPECIFIC OBJECTIVES

- To detect the presence and determine the prevalence of CMV, HPV and HCV.
- To determine the distribution of HPV genotypes in Northern South Africa.

CHAPTER 2: MATERIALS AND METHODS

2.1. ETHICAL CLEARANCE

The current study was covered by ethical approval from the University of Venda Health, Safety and Research Ethics Committee (SMNS/09/MBY/003 and SMNS/11/MBY/001). The samples were de-identified for the current purpose.

2.2. STUDY SITE AND DEMOGRAPHIC DATA

Study participants were recruited from Bela Bela in Capricorn district of Limpopo Province. Bela Bela is a holiday resort with hotels and camping sites. It is also a stop-over point for long distance truck drivers who patronize sex workers attracted to the luxury facilities of the area. It is surrounded by localities of poor socioeconomic status. Other participants were from Venda and Mankweng. The study comprised of two hundred HIV positive and fifty HIV negative individuals that were chosen randomly according to age and gender. The age range was from 2 to 68 years which gives a mean of 35 years. Some of the HIV infected participants were on treatment and others were co-infected with tuberculosis.

2.3. HPV, CMV and HCV ELISA

Enzyme-linked immunosorbent assay for cytomegalovirus, human papillomavirus and hepatitis C virus was done for IgG antibody detection using the kits according to the manufacturers' instructions. HCV ELISA (Immunodiagnostics) was a qualitative indirect method for detection of IgG antibodies to HCV with a two-step incubation procedure. The results were calculated by relating each sample OD value to the Cut-off value which was calculated using the following formula C.O. = Nc + 0.12, where Nc is the mean absorbance value for the three negative controls. Samples that had an OD value less than Cut-off were considered negative and those which were greater than the Cut-

off were positive. Samples with OD less than C.O. X 2 were considered borderline and were retested in duplicates.

DRG HPV ELISA kit used was an indirect ELISA method for the semi-quantitative determination of IgG class antibodies to HPV in human plasma. Results were calculated according to the following formula: Cut-off = Nc + 0.250. Sample OD value that was lower than the C.O. was considered negative and if greater, then it was considered positive.

Ab108724-Anti-Cytomegalovirus (CMV) IgG Human ELISA Kit (Abcam) used was an indirect qualitative method that detects and measures the presence of IgG antibodies against cytomegalovirus in human plasma or serum. The assay is 97.5% specific and 98% sensitive. The absorbance of the IgG antibodies was measured at 450nm wavelength and the results were considered positive when the sample absorbance value was greater than 10% of the Cut-off value. Samples with an absorbance value less than 10% above or below the Cut-off control value were considered as inconclusive and retested. Samples with an absorbance value lower than 10% below the Cut-off were considered negative.

2.4. DNA EXTRACTION

DNA was extracted from 200µl plasma using the QiAamp MinElute Virus Spin from Qiagen following the manufacturer's instructions.

2.5. POLYMERASE CHAIN REACTION

(a) Linear Array HPV Genotyping Test (Roche)

The polymerase chain reaction was achieved by the use of Linear Array HPV Genotyping Test (Roche). The kit consisted of master mix, magnesium ions, positive and negative controls. The master mix contained the primers for the amplification of DNA from 37 HPV genotypes and the human β-globin gene. Biotinylated consensus

HPV PCR primer pair and was used to amplify a 450bp of the L1 region of the Human Papillomavirus. The human β-globin gene of 268bp was also amplified. The cycling conditions included Amperase activation at 50°C for 2 minutes, Taq activation at 95°C for nine minutes, forty cycles of 95°C for 30 seconds, 55°C for a minute, 72°C for a minute, final extension at 72°C for five minutes and held indefinitely at 72°C.

(b) Conventional PCR

A protocol was designed and optimized to amplify HPV DNA in order to obtain a 450bp fragment of HPV partial L1 gene using consensus primers MY11 5' GCA CAG GGA CAT AAC AAT GG 3' and MY09 5' CGT CCC AAA GGA AAC TGA TC 3' in a one tube nested PCR containing the following reagents: 2,5µl of 10X PCR buffer, 0.25µl of 20mM dNTP mix, 0.2µl of 0.5U/µl Taq Polymerase, 1.5µl of 50mM magnesium chloride, 0.5µl of10µM MY09, 0.5µl of 10µM MY11, 2µl of DNA template and 17.55µ of PCR water to make a reaction volume of 25µl. The cycling conditions included Taq activation at 95°C for 3 minutes, forty cycles of 95°C for 30 seconds, 51°C for 30 seconds, 72°C for a minute and final extension at 72°C for 5 minutes.

2.6. AGAROSE GEL ELECTROPHORESIS

The amplified products from PCR were detected and verified for size, by running a (2%) agarose gel. A 2% agarose gel was prepared by weighing 1g of agarose powder and mixed with 50ml of 1X TAE buffer. The solution was dissolved in the microwave oven (high) for three minutes. The gel solution was cooled to 40°C and 3µl of ethidium bromide was added. The comb was inserted to create the wells, gel was poured on the tray and left to solidify at room temperature. After solidification, the comb was removed and the tray was placed in the electrophoresis chamber. The parafilm paper was used to mix the samples with the loading dye and the ladder. After mixing, the samples were loaded into the wells. The lid was placed on the electrophoresis chamber with the negative terminal at the same side with the wells. The electrophoresis box was set to

run at 80V for one hour. After an hour, the gel was viewed under a UV transilluminator (G-Box) from Syngene and the gel photo was captured by the Genesnap program.

2.7. GENOTYPING OF HPV

Human Papillomavirus genotyping was done by hybridization and detection reaction using Linear Array Detection kit (Roche) following manufacturer's instructions. The kit determines 37 high and low risk genotypes. Briefly, amplicons were chemically denatured to form single-stranded DNA by the addition of denaturation solution immediately after polymerase chain reaction and were hybridized to an immobilized HPV probe array strip from Roche. The HPV type was identified by the detection reaction where the strip was coated with probes that the amplicon was hybridizing to.

2.8. SEQUENCING OF HPV PARTIAL L1 GENE

The HPV DNA was sequenced using direct population-based method and it was performed on both strands with the Big Dye terminator kit on ABI Prism 377 (Applied Biosystems). Primers used for sequencing were the same as the ones used for DNA amplification with the following sequences: MY11 5' GCA CAG GGA CAT AAC AAT GG 3' and MY09 5' CGT CCC AAA GGA AAC TGA TC 3' Sequences were edited using SeqMan and the nucleotides were aligned using BioEdit.

2.9. PHYLOGENETIC ANALYSIS OF HPV

Human Papillomavirus types were determined by the phylogenetic tree analysis where ClustalX programme and the neighbor-joining method were used to generate the phylogenetic tree.

2.10. STATISTICAL ANALYSIS

Statistical analysis was done to check for significant differences in the distribution of infection between age and gender. The statistical analysis was done using SPSS version 22 software and the p-value was calculated using Chi-square where $p<0.05$ was considered significant at 95% confidence interval.

CHAPTER 3: RESULTS

3.1. DEMOGRAPHIC DATA OF STUDY PARTICIPANTS

A total of 250 samples were used for this study. The study participants comprised of 200 HIV positive and 50 HIV negative individuals. Out of 250 participants, 161 (64%) were females, 72 (28.8%) were males and 17 (6.8%) did not mention their gender. The population mean age was 35 (2-68 years). Sixty five point six percent of individuals were single, 20% were married, 1,6% were divorced, 10.8% did not mention and 2% were widowed. Twenty one point six percent were between the age of 1-25 years, 61.6% were between 26-50 years, 6% were from 51-70 years and 10.8% had no data available. Nine HIV positive subjects were also co-infected with tuberculosis, 7 were females and 2 were males. The resuslts are shown in table 1.

Table 1: Demographics of the study participants

Gender	**No. (%)**
Female	161 (64)
Male	72 (28.8)
Data not available	17 (6.8)
Age	**No. (%)**
1- 25 years	54 (21.6)
26-50 years	154 (61.6)
51-70 years	15 (6)
Data not available	27 (10.8)
Marital status	**No. (%)**
Single	164 (65.5)
Married	50 (20)
Divorced	4 (1.6)
Widow	5 (2)
Data not available	27 (10.8)
Tuberculosis	**No. (%)**
Female	7 (2.8)
Male	2 (0.8)

3.2. PREVALENCE OF ANTIBODIES TO HCV, HPV AND CMV

The study comprised two hundred and fifty patients. Two hundred were HIV infected individuals and fifty were HIV negative individuals. The antibody prevalence of Hepatitis C virus, Human papillomavirus and Cytomegalovirus was 0.05% (1/200), 21% (42/200) and 100% (200/200) in HIV infected population and 0% (0/50), 12% (6/50) and 100% (50/50) in HIV negative group respectively. Infection with HCV was very low in HIV infected population. Infection rate with HPV was not significant in HIV infected females (p=1.000) when compared with the HIV negative group. Similarly, there was no significant difference of HPV infection when age below 15 years and above was considered in the HIV infected and non-infected groups (p=0.983). The CMV infection was significantly more prevalent among the unmarried patients than married patients (p=0.000). Eight percent (16/200) HIV infected people were found to be co-infected with both HPV and CMV. For HPV ELISA, 48 samples were reactive and 42 samples were positive from HIV infected individuals and 6 were from HIV negative samples. There were 31 females, 12 males and 5 with no gender data available. The results are shown in table 2, figures 11 and 12.

Table 2: Demographic data of HPV seroprevalence

HIV status	Gender	No. (%)
Positive	Female	27 (64.3)
	Male	10 (23.8)
	Data not available	5 (11.9)
	Age (years)	**No. (%)**
	2-25	6 (14.3)
	26-50	28 (66.6)
	51-70	1 (2.38)
	Data not available	7 (16.6)
Negative	**Gender**	**No. (%)**
	Female	4 (1.6)
	Male	2 (0.8)
	Age (years)	**No. (%)**
	2-25	3 (1.2)
	26-50	3 (1.2)

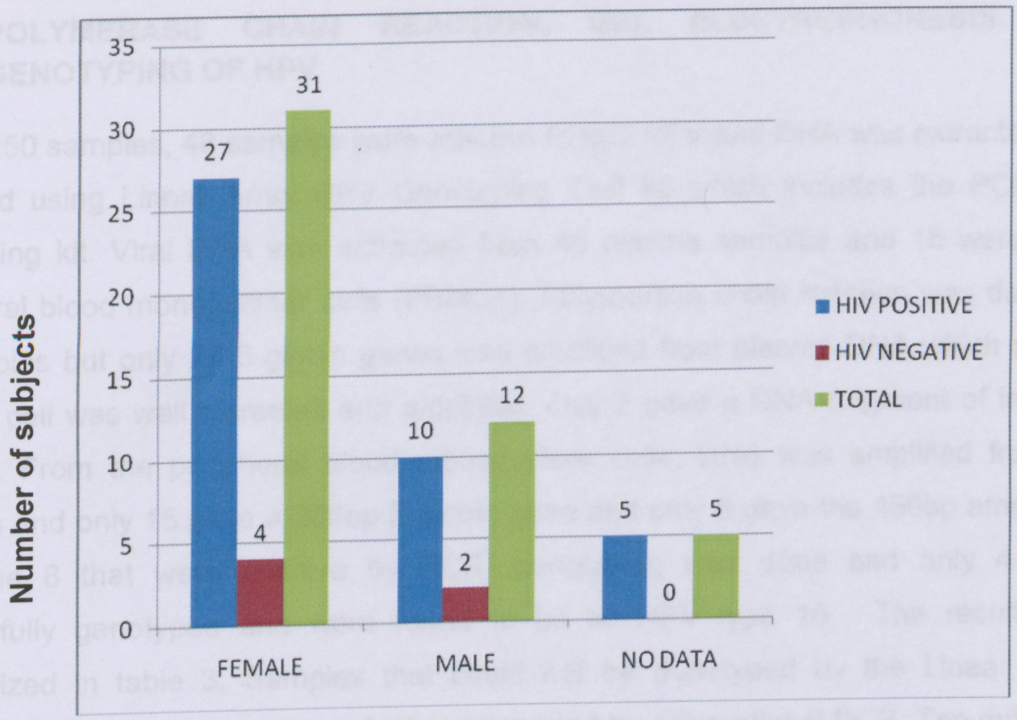

Figure 11: HPV distribution between genders in HIV positive and negative individuals.

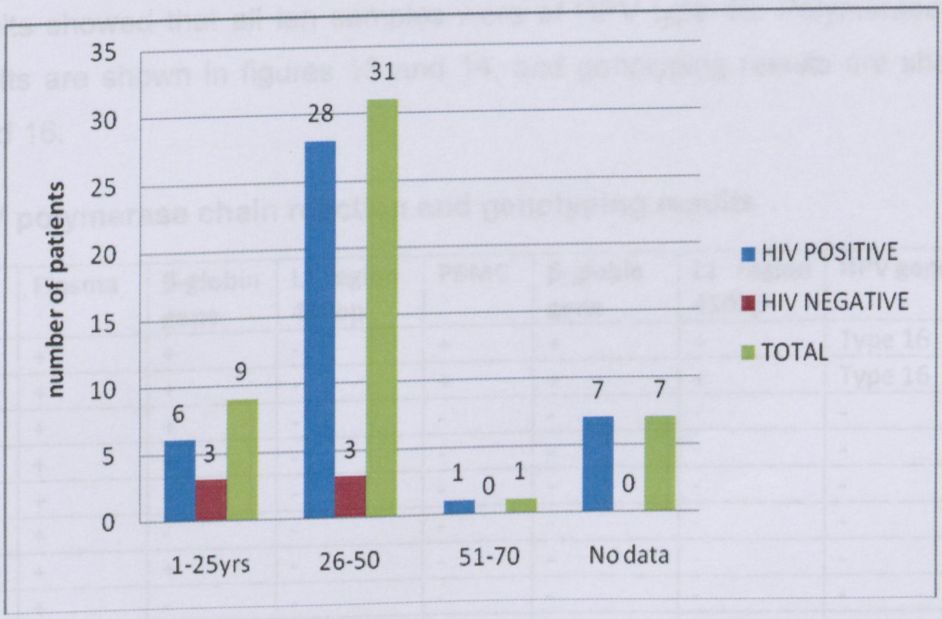

Figure 12: Graph showing HPV distribution among different age groups

3.3. POLYMERASE CHAIN REACTION, GEL ELECTROPHORESIS AND GENOTYPING OF HPV

Out of 250 samples, 48 samples were reactive to IgG HPV and DNA was extracted and amplified using Linear Array HPV Genotyping Test kit which includes the PCR and genotyping kit. Viral DNA was extracted from 48 plasma samples and 16 were from peripheral blood mononuclear cells (PBMCs). Polymerase chain reaction was done on 48 samples but only 27 β-globin genes was amplified from plasma DNA which shows that the cell was well extracted and amplified, only 2 gave a DNA fragment of interest (450bp). From the peripheral blood mononuclear cells, DNA was amplified from 16 samples and only 15 gave a 268bp β-globin gene and only 8 gave the 450bp amplicon. From the 8 that were positive by PCR, genotyping was done and only 4 were successfully genotyped and were found to be all HPV type 16. The results are summarized in table 3. Samples that could not be genotyped by the Linear Array Genotyping Test kit (Roche) were further attempted by conventional PCR. Ten out of 16 samples were positive yielding a 450bp. The amplicons were directly sequenced and phylogenetic analysis was done to determine the HPV viral type. The phylogenetic analysis results showed that all ten samples were of HPV type 16. Polymerase chain reaction results are shown in figures 13 and 14, and genotyping results are shown in figures 15 and 16.

Table 3: HPV polymerase chain reaction and genotyping results

Sample code	Plasma	β-globin gene	L1 region 450bp	PBMC	β-globin gene	L1 region 450bp	HPV genotype
MB 011	+	+	-	+	+	+	Type 16
MB 015	+	+	-	+	+	+	Type 16
MB 09-23	+	+	-	-	-	-	-
MARBB 01	+	+	-	-	-	-	-
MARBB 15	+	+	-	-	-	-	-
MAD 051	+	-	-	-	-	-	-
BBVCT 2010/20	+	+	-	-	-	-	-
SEPI 04	+	-	-	-	-	-	-
SEPI 08	+	-	-	-	-	-	-
SEPI 15	+	-	-	-	-	-	-
SEPI 33	+	-	-	-	-	-	-

Sample code	Plasma	β-globin gene	L1 region 450bp	PBMC	β-globin gene	L1 region 450bp	HPV genotype
SEPI 35	+	+	-	-	-	-	-
SEPI 38	+	-	-	-	-	-	-
MC 017	+	+	+	+	+	+	-
MC 020	+	+	-	+	+	+	Type 16
MC 050	+	+	-	+	+	+	Type 16
MC 012	+	+	-	+	+	-	-
MC 039	+	-	-	-	-	-	-
MARBB 54	+	+	-	-	-	-	-
MARBB 55	+	+	-	-	-	-	-
MARBB 58	+	-	-	-	-	-	-
BBVCT 2010/21	+	+	-	-	-	-	-
BBVCT 2010/23	+	+	-	-	-	-	-
MARBB 39	+	+	-	-	-	-	-
MARBB 40	+	-	-	-	-	-	-
MARBB 63	+	-	-	-	-	-	-
MARBB 66	+	-	-	-	-	-	-
MARBB 67	+	-	-	-	-	-	-
MB 020	+	+	-	+	+	-	-
BBVCT 71	+	+	-	-	-	-	-
MC 008	+	-	-	+	+	-	-
MC 035	+	-	-	-	-	-	-
MC 029	+	-	-	-	-	-	-
MC 028	+	-	-	+	+	-	-
BBVCT 69	+	-	-	+	-	-	-
MC 032	+	+	-	+	+	-	-
BBVCT 48	+	+	-	-	-	-	-
UNIV 2/2011	+	-	-	-	-	-	-
MB 033	+	-	-	+	+	+	-
MC 043	+	+	-	+	+	-	-
BBVCT 72	+	+	-	-	-	-	-
UNIV 5/2011	+	+	-	-	-	-	-
MAD 049	+	-	-	-	-	-	-
MB 034	+	+	-	+	+	+	-
BBCR 060	+	+	-	+	+	-	-
MB 030	+	+	-	+	+	+	-
MARBB 61	+	-	-	-	-	-	-
BBVCT 70	+	+	+	-	-	-	-

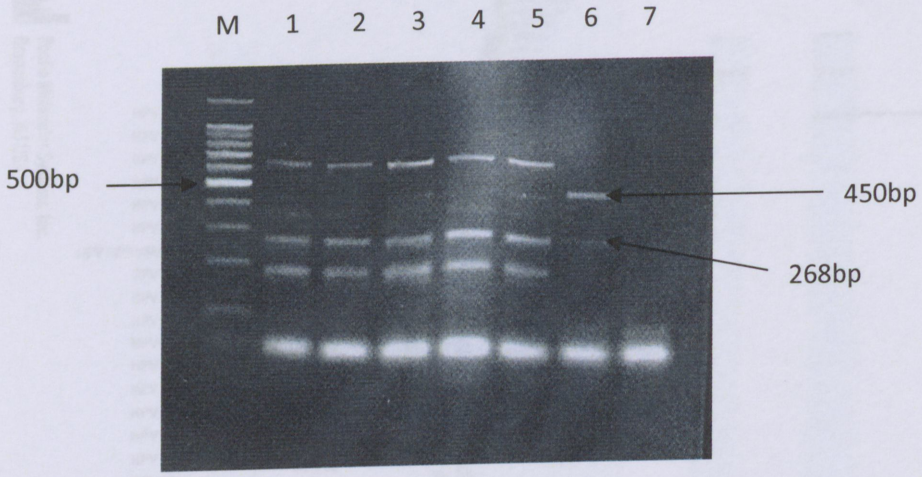

Figure 13: Gel electrophoresis photo showing a 268bp β-globin gene and 450bp amplified HPV DNA. Lane M represent 100bp ladder, lanes 1-5 represent samples, lanes 6 and 7 represent positive and negative controls respectively.

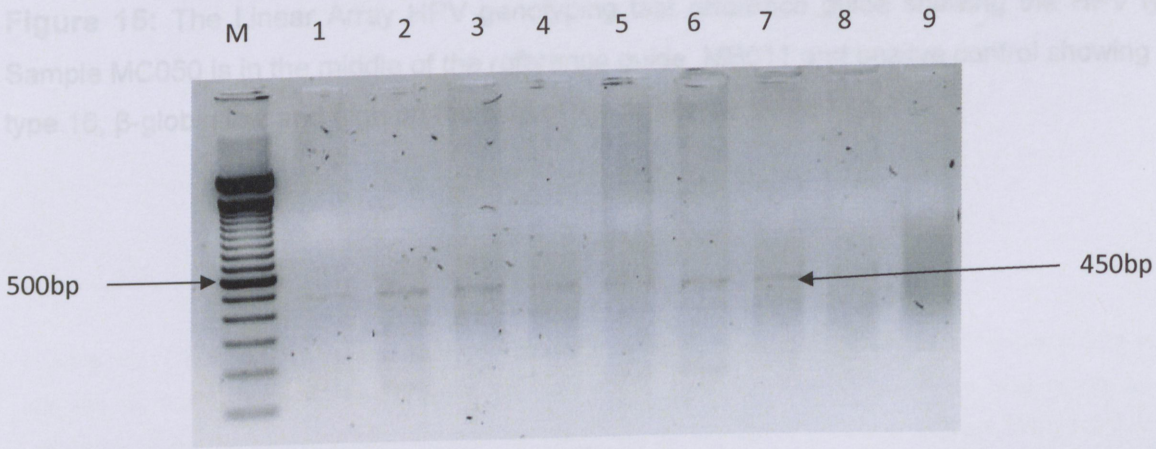

Figure 14: Gel electrophoresis showing a 450bp fragment of HPV DNA. Lane M represents 100bp ladder, lanes 1- 8 represent samples and lane 9 represents a negative control.

3.4. HUMAN PAPILLOMAVIRUS GENOTYPING RESULTS

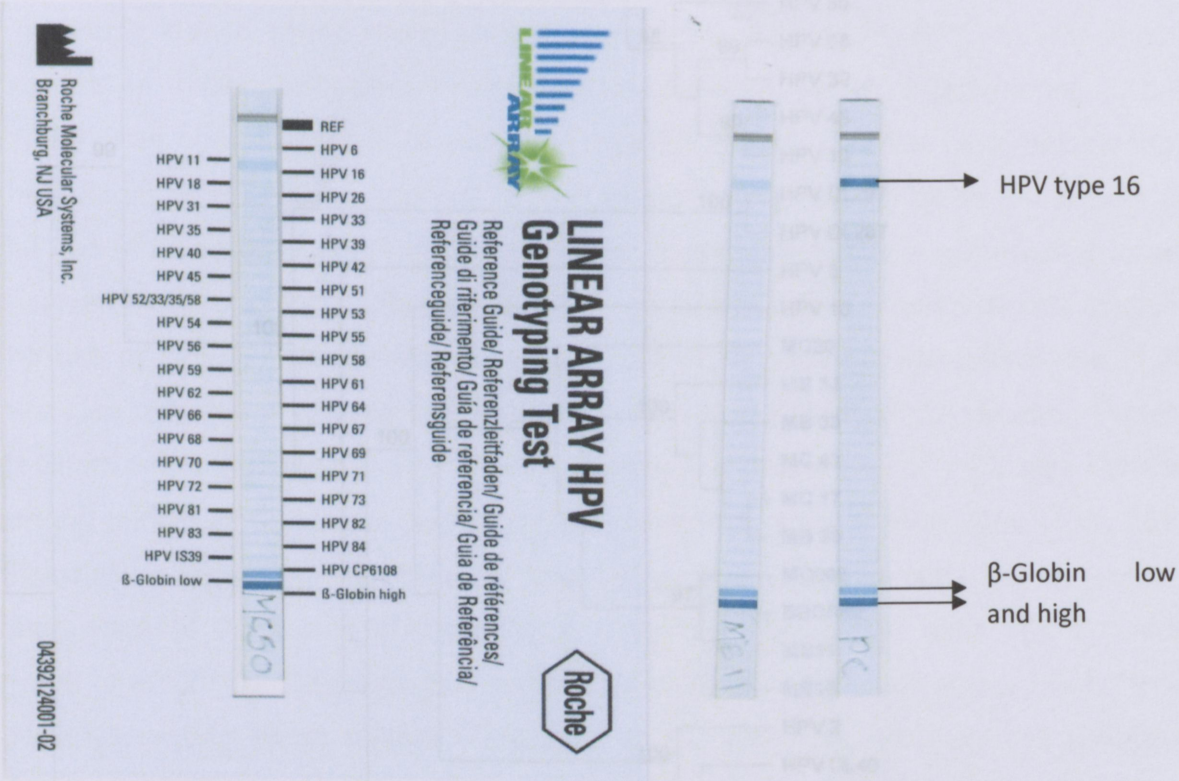

Figure 15: The Linear Array HPV genotyping test reference guide showing the HPV types. Sample MC050 is in the middle of the reference guide, MB011 and positve control showing HPV type 16, β-globin low and high on the side of the reference guide.

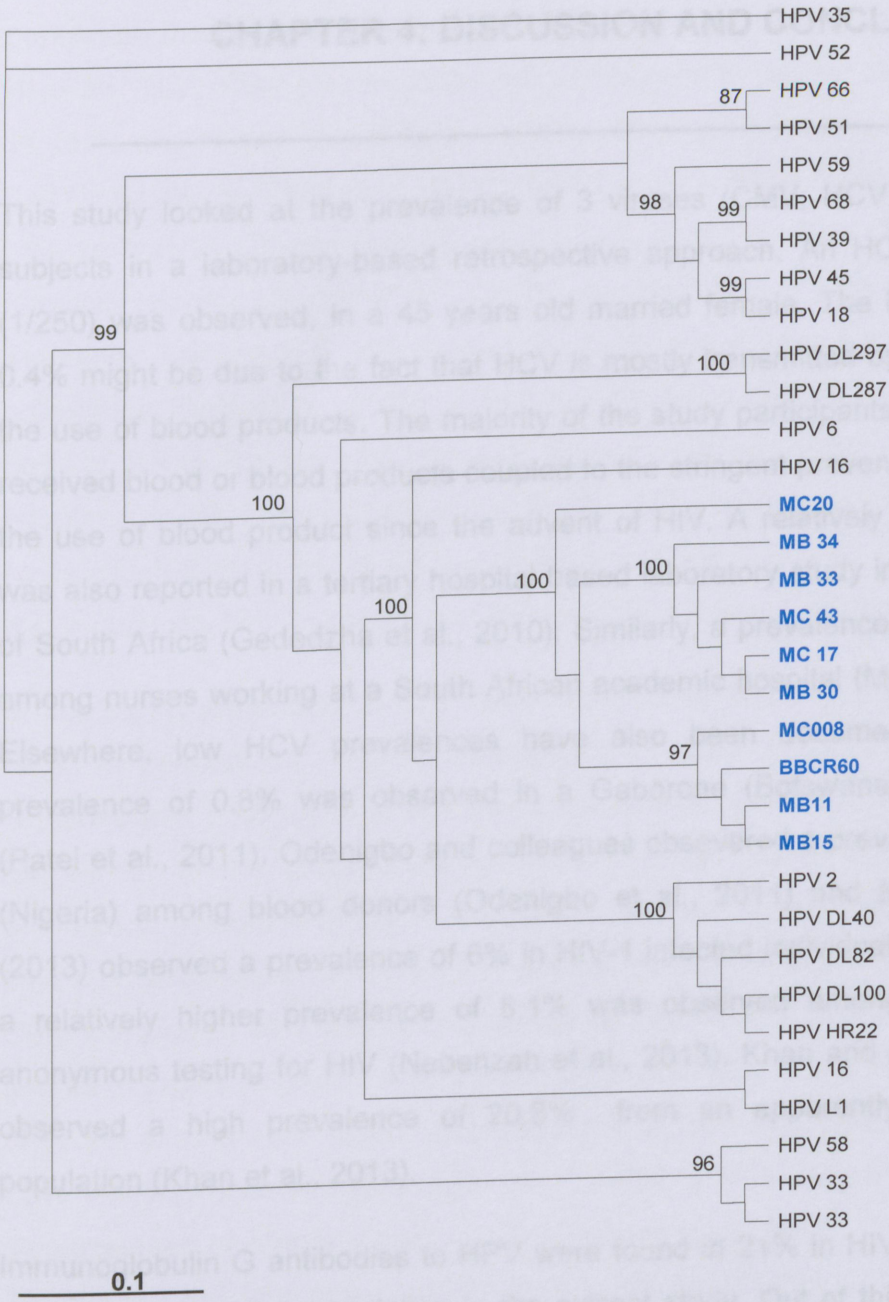

Figure 16: Phylogenetic relationship of test sequences (in blue) and reference sequences. The test sequences form 2 sub-clusters closely related to HPV type 16. The viruses were also typed as of genotype 16 by the Linear Array Genotyping Test. This shows that the sequences are likely HPV type 16. The sequences were aligned and the phylogenetic tree was generated by the neighbor-joining method in ClustalX. References sequences were obtained from the GenBank and the Virus Sequence Database. Bootstrap values above 70% are shown.

CHAPTER 4: DISCUSSION AND CONCLUSION

This study looked at the prevalence of 3 viruses (CMV, HCV, HPV) in HIV infected subjects in a laboratory-based retrospective approach. An HCV prevalence of 0.4% (1/250) was observed, in a 45 years old married female. The low HCV prevalence of 0.4% might be due to the fact that HCV is mostly transmitted by blood transfusion and the use of blood products. The majority of the study participants were reported to have received blood or blood products coupled to the stringent prevention methods applied in the use of blood product since the advent of HIV. A relatively low prevalence (1.2%) was also reported in a tertiary hospital based laboratory study in the Gauteng Province of South Africa (Gededzha et al., 2010). Similarly, a prevalence of 1.8% was observed among nurses working at a South African academic hospital (Mosendane et al., 2012). Elsewhere, low HCV prevalences have also been documented. For example, a prevalence of 0.8% was observed in a Gaborone (Botswana) adult HIV population (Patel et al., 2011). Odenigbo and colleagues obsevered a prevalence of 2% in Nnewa (Nigeria) among blood donors (Odenigbo et al., 2011) and Muriuki and colleagues (2013) observed a prevalence of 6% in HIV-1 infected individuals in Nairobi. In Angola, a relatively higher prevalence of 8.1% was observed among individuals attending anonymous testing for HIV (Nebenzah et al., 2013). Khan and colleagues in Pakistan, observed a high prevalence of 20.8% from an apparently healthy young male population (Khan et al., 2013).

Immunoglobulin G antibodies to HPV were found in 21% in HIV- infected patients and 12% in HIV negative population in the current study. Out of this, 10 samples (20.8%) were genotyped for HPV by phylogenetic analysis and were found to be HPV type 16 which is a high risk HPV type for cervical cancer. HPV infection was three times higher in females (64.5%) than males (20.8%) in the HIV infected group. Similar results were also observed by Ritcher and colleagues where HPV type 16 was detected in 19.5% of women (Richter et al., 2013). The HPV IgG prevalence was higher in HIV infected

population than in HIV negative group. Even though there was a relationship between HPV and HIV, there was no statistical significance in gender, and the age group below 14 years and above. Also in Benin, a prevalence of 17.6% was observed in women who had HPV type 16 (Piras et al., 2011). In India, Sarkar and colleagues observed the HPV type 16 and 18 with the prevalence of 32.2% in HIV positive females and 9.1% in HIV negative females (Sarkar et al., 2011). A prevalence of 23% of HPV type 16 among Tanzanian women and 34% among South African women was observed by Dols and colleagues (Dols et al., 2012). A higher prevalence of HPV type 16 was observed in 56% of South African HIV negative women with and without cervical intraepithelial neoplasia in 2012 (McDonald et al., 2012). HPV type 16 is one of the high risk HPV types which is included in the quadrivalent vaccine Gardasil. The current study shows that a relatively high prevalence of HPV infection is found in Northern South Africa.

In this study, antibodies to CMV was found to be 100% in both the HIV positive and negative populations. The seroprevalence of CMV is generally high in developing countries and among those of lower socio-economic status. The results reported here are similar to the study done by Neirukh et al. (2013) where they found a high prevalence of 96,6% of CMV in pregnant women, 88% of hospitalized children and 98,4% of hospitalized newborns in Palestine (Neirukh et al., 2013). A high prevalence of CMV infection of 96% was also observed by Akinbami et al., (2009) from healthy blood donors in Lagos (Nigeria). Also, Chaudhari and Bindra (2009) found a CMV prevalence of 87,9% among male blood donors in India. In Northern India, a prevalence of 87.1% CMV IgG was observed (Bhargava et al., 2009). A high CMV prevalence of 80.7% was also observed in Jeddah Saudi Arabia (Redwan et al., 2011). In Sudan, a prevalence of 72.2% CMV IgG was observed in pregnant women in 2011 by Hamdan and colleaques (Hamdan et. al., 2011). A lower prevalence was found by Stadler et al., (2010) where a prevalence of 47% was seen in adolescent males in African American race (Stadler et al., 2010). Odland and colleagues detected 59.9% of CMV IgG antibodies in pregnant Norwegian women (Odland, 2013). South Africa has a high burden of HIV disease and a large, expanding HAART programme. The fact that most South African HIV-infected

patients prior to 2012 initatied HAART when their CD4+ cell count was less than 350 cells/mm^3 could have made them far more susceptible to CMV infection and the prevalence could be high in these patients. However, HAART has improved the survival rate of AIDS CMV-retinitis patients since its introduction in South Africa (Stewart, 2010).

Limitations of the current study include: The small number of patients investigated which limits extrapolation of findings in an epidemiological context. Cervical cells are expected to give a better HPV DNA yield for subsequent genotyping investigations. However, in the current study the sample types available for HPV genotyping were plasma and peripheral blood mononuclear cells which provided a lower DNA yield. However, this is a first report of HCV, HPV and CMV in an HIV infected population in northern South Africa. Further studies need to be conducted using a large sample size in order to establish the statistics that can be conclusive and also give a good indication on how prevalent a particular infection is in the HIV infected population.

In conclusion, among the HIV co-infections that were detected in this study, CMV was found to be more prevalent in both HIV positive and HIV negative groups irrespective of gender and age. Human papillomavirus was more prevalent in women than men with type 16 apparently more common. Hepatitis C is apparenlty low in the population. With HPV, the use of Gardasil vaccine which prevents against four types of HPV can be effective since the prevalent type observed was HPV type 16 which is a high-risk type for cervical cancer.

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