Identification of human papilloma virus, hepatitis B virus and human herpes virus type 8 in plasma of benign prostatic hyperplasia and prostate cancer patients in South Africa

By

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Declaration

I, Munzhedzi Mukhethwa hereby declare that the dissertation for a MSc degree in Microbiology at the University of Venda, hereby submitted by me has not been submitted previously for the degree at this or other university, that it is my own work in design and execution, and all the references contained therein have been duly acknowledged.

Signature ---------- Date----------

Candidate: Munzhedzi Mukhethwa

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This dissertation has been made possible by the following people / institutions:

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ABSTRACT

Background: Prostate cancer (PCA) is a major health concern in males, particularly those above 40 years old. It is the most common form of cancer in males worldwide, including South Africa. In South Africa, the rate of histologically diagnosed prostate cancer is 40 per 100 000 in whites and 14 per 100 000 in blacks, and 1 in 8 men will develop PCA in their lifetime. Several reports have suggested the association of viruses in the pathogenesis of prostate cancer.

Objectives: This study was aimed at identifying Hepatitis B virus (HBV), human papilloma virus (HPV) and human herpes virus type 8 (HHV-8), implicated in other forms of cancer, in a cohort of South African patients with either PCA or benign prostatic hyperplasia (BPH); and to seek possible associations thereof.

Methods: The study group comprised 187 male patients recruited from Polokwane Hospital presenting with either PCA (staged by Gleason scores) or BPH. Enzyme-linked immunosorbent assay was used to detect antibodies to HHV-8 and HPV; and to detect hepatitis B surface antigen (HBsAg) in the plasma of the study subjects. Total DNA was extracted from plasma and targeted for the identification of HBV and HHV-8 DNA by nested PCR protocols. The HBV nested PCR protocol amplifies a 336bp fragment of the overlapping surface polymerase gene of HBV. The HHV-8 nested protocol amplifies a 233bp fragment of the ORF 26 gene of HHV-8. Amplified DNA products were purified, sequenced by the Sanger protocol and phylogenetically analysed for viral genotypes. The Chi-square test was used to infer statistically significant differences in the level of detection of viruses and the stage of prostate cancer development.

Results: Of the 187 participants, a seroprevalence of 4.8% (9/187, HBsAg), 5.3% (10/187, HPV IgG antibody) and 27% (33/124, HHV-8 IgG antibody) were observed. HBsAg was detected more in individuals with BPH than those without and this was statistically significant at (χ^2=6.0, p< 0.05). HHV-8 DNA was detected more in individuals in the 60-79 years age range and this was statistically significant at (χ^2=61.1, p< 0.05). Occult HBV infection (that is the presence of HBV DNA in the absence of HBsAg) was detected in 23/178 (12.9%) of patients. Taking into account occult HBV infection, the overall prevalence of HBV was 17.7%. HBV genotype E was more prevalent (86.7%) followed by genotype A (13.3%). HHV-8 genotypes K and R were inferred. Apparently, this is the first report on the identification of HHV-8 genotypes K and R from South Africa.

Conclusion: The current study has demonstrated for the first time, the presence of genotypes K and R of HHV-8 in South Africa. This study also suggests that there is a high

level of occult genotype E HBV infection. Future studies will explore the virome in prostate cancer biopsies.

Keywords: HPV; HBV; HHV-8; Prostate cancer; Benign prostatic hyperplasia.

Dedication

This dissertation is dedicated to God Almighty,

my dad (Prophet Munzhedzi TE) and my mom (Munzhedzi LP), thank you.

Ndiya vha funa. Aa.

Contents

List of abbreviations

ABI	Advanced Biotechnologies Incorporation
AIDS	Acquired immunodeficiency Syndrome
AKS	Acquired immunodeficiency Syndrome associated with Kaposi's sarcoma
BPH	Benign prostatic hyperplasia
CCC DNA	Covalently closed circular Deoxyribonucleic Acid
CCP	Complement control protein
CKS	Classical Kaposi's sarcoma
E1	Early gene 1
E2	Early gene 2
ER	Endoplasmic Reticulum
FADD	Fas-associated death domain
FLICE	FADD like IL-beta converting enzyme
HBV	Hepatitis B Virus
HHV-8	Human Herpes Virus-8
HIV	Human immunodeficiency virus
HPV	Human Papilloma Virus
IKS	Iatrogenic Kaposi's sarcoma
kb	Kilo base
KS	Kaposi's sarcoma
L1	Late gene 1
L2	Late gene 2
LANA	Latency associated nuclear antigen
LCR	Long coding region
ORF26	Open reading frame 26
PCA	Prostate cancer
PSA	Prostate Specific Antigen
SAPCS	Southern African Prostate Cancer Study

URR	Upstream regulated region
V-CYC	Viral D-type cyclin
V-flip	Viral FLICE inhibitory protein
Vgpcr	Viral G-protein coupled receptor
vIL-6	Viral interleukin 6
vMIP	Viral macrophage inflammatory protein
WHO	World Health Organisation

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CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Prostate cancer (PCA) develops from cells in the prostate gland, an organ in the male genital system. Prostate cancer is of grave concern for all men as it poses a health threat especially to men over the age of 40 (Modeste *et al*., 2003; Arafa *et al*., 2015). It is the most common male cancer occurring globally (Velonas *et al*., 2013; Babb *et al*., 2014). It is the leading cancer in men of African descent in the United States of America (USA), the Caribbean and Sub-Saharan Africa (Rebbeck *et al*., 2013). In the USA, PCA is the second leading cause of death related to cancer in men (Moul, 2004) and the sixth cause of mortality among men globally (Ghasemzadeh *et al*., 2014). There were approximately 220 000 new diagnoses projected for 2015 (Velonas *et al*., 2013, Izumi *et al*., 2015 and Mofolo *et al*., 2015). The global burden of new cases of prostate cancer is expected to reach 1.7 million and 499 000 deaths by 2030 (Jain *et al*., 2014). This burden is, however, not equally distributed around the world, as the developed countries register the highest incidence rates (Farley *et al*., 2010 and Tindall *et al*., 2014). The worldwide incidence of PCA also varies among different geographical regions and or ethnic groups (Heyns *et al*., 2011) but little is known about PCA in Africa (Babb *et al*., 2014).

In South Africa, (Free State Province, Bloemfontein), the rate of histologically diagnosed prostate cancer is 40 per 100 000 in whites and 14 per 100 000 in blacks (Parkin *et al*., 2005; Mofolo *et al*., 2015). Since blacks have limited access to diagnostic facilities, they often develop an aggressive type of prostate cancer. Southern African men present a prostate cancer incidence at 40.5 per 100 000 of the population per year with a mortality rate of 22.5 per 100 000 per year (Mofolo *et al*., 2015). About one in 8 men will develop PCA in their lifetime (Kgatle *et al*., 2016). Although the cause of prostate disease is unknown, several risk factors exist which contribute to the development of the disease (Michopoulou *et al*., 2014, Araujo-Neto *et al*., 2016). Non-modifiable risk factors of prostate cancer include old age, family history and African ancestry (Bae, 2015). Known modifiable factors include environmental factors (viruses, bacteria), smoking, drinking, lack of exercise and diet (McDonald *et al*., 2011; Ghasemzadeh *et al*., 2014).

Studies suggest that infectious agents may be one of the significant preventable causes of cancer. Viruses play an important role in cancer biology by shedding light in our understanding of cell signaling as well as growth control pathways that result in cancer. This has been possible through the study of viral transforming properties. Viruses have been consequently suggested to be causative agents of human neoplasia. Previous studies have

shown that 17.8% of cancer cases are attributable to infectious agents of which 12.1% correspond to viral infections (Carrillo-infante *et al*., 2007). A more recent study estimated that viral infections contribute 15-20% to all human cancers (Jha *et al*., 2016). Viruses act as oncogenes to develop disease through different mechanisms. There is limited data on the association of PCA and viruses specifically Human Papilloma virus (HPV), Hepatitis B virus (HBV) as well as Herpes virus type 8 (HHV-8). The role that these viruses play in prostate cancer is not well understood, however, it has been suggested that they (HPV and HHV-8) play a role in pathogenesis of PCA (Ge *et al*., 2013).

Epidemiological evidence is accumulating that sexual history (e.g. number of sexual partners) may be related to prostate cancer (Adami *et al*., 2003). It has been established from some studies that a relationship exists between HPV and PCA (Adami *et al*., 2003; Al-Maghrabi, 2007). For example, HPV has been suggested to play a role in the pathogenesis of prostate cancer (Al-Maghrabi, 2007 and May *et al*., 2008). A relationship or link has also been suggested for HBV and PCA (Ishiguro *et al*.,2017). It has also been reported that HHV-8 plays a significant role in the pathogenesis of cancer (Ge *et al*., 2013). This happens through the inhibition of cell apoptosis and stimulation of DNA synthesis which may finally lead to cancer.

This study intends to shed light on HBV, HPV, and HHV-8 implicated in other forms of cancer with either PCA or benign prostatic hyperplasia (BPH); and to seek possible associations thereof.

1.1.1 Prostate cancer disease burden and distribution in South Africa

A study done in South Africa by Babb *et al*., (2014) found PCA to be the most commonly diagnosed cancer in males (based on pathological samples) (http://www.canceralliance.co.za). It is the most prevalent cancer among South African White males, however, recent statistics show that Black males are at an increased risk of PCA and often develop an aggressive type resulting from the lack of PSA screening (diagnostic facilities) in SA (CANSA, fact sheet 2016). According to Tindall *et al*., (2014), men in Limpopo Province present almost 3 years later than what is found in other parts of the world. PCA is the most common cause of death in all men in SA (Herbst, 2016). Southern African men present a PCA incidence at 41 per 100 000 of the population per year with a mortality rate of 23 per 100 000 per year (Mofolo *et al*., 2015). Since PCA is often asymptomatic, it is more difficult to detect, therefore regular screening for PCA is essential. It's however extremely rare before the age of 40 years (Powel, 2012).

1.1.2 Prostate cancer management in South Africa

Early detection and intervention is the greatest hope of minimizing PCA (Tindall *et al*., 2013). Cancer that is localized to the prostate gland is curable by radical prostactomy or radiotherapy; however the benefit of the cure will only be apparent in about 10 years. This is due to the slow growing nature of prostate cancer. There is currently no cure for meatstatic disease (Tindall *et al*., 2013); however it can be controlled by (hormonal treatment) depriving the cancer of male hormone (Testosterone) which are responsible for the differentiation and maturation of the male sexual organs and secondary sexual characteristics. Testosterone can be converted to more potent hormones, either estradiol by aromatization or dihydrotestosterone (DHT) by the 5-alpha- reductase enzyme found in many androgen target tissues such as the prostate. DHT is much more potent as compared to testosterone in promoting prostate growth (Roth and Page, 2011). With respect to the severity of the disease, the current therapies include watchful waiting, hormone therapy, therapeutic vaccines, bone directed treatment, cryotherapy, radiation therapy and surgery. These options may improve the quality of life of patients by delaying or inhibiting disease progression; chemotherapeutic resistance commonly develops and often results in death (Kgatle *et al*., 2016). South Africa lacks the necessary population based screening for prostate cancer like many African countries (Matshela *et al*., 2014).

1.1.3 Screening and diagnostic techniques for prostate cancer

These screening and diagnostic techniques include digital rectal examination (DRE), urine testing and Prostate Specific Antigen (PSA) measurement. The DRE involves the insertion of a lubricated, gloved finger into the rectum to examine the prostate. The urine test checks for the presence of blood in urine. The PSA measurement measures the PSA level in blood. Since the prostate normally secretes small amounts of PSA, a higher level may indicate prostatatic disease (Herbst, 2016). This may be cancer, an enlarged prostate caused by infection or benign prostatic hyperplasia

The more specific tests include transrectal ultrasound which involves the insertion of a probe into the rectum to check the prostate for abnormal areas; and transrectal biopsy which involves the removal of tissue to histologically examine for cancer cells (Herbst, 2016) and the staging (Gleason). It is the only sure way to diagnose prostate cancer.

1.1.4 Human Papilloma Virus biology

Human papilloma virus (HPVs) is a small, non-enveloped, double stranded circular, icosahedral, deoxyribonucleic acid (DNA) virus (Peevor and Fiander, 2010; Stanley, 2010)

belonging to the Papillomaviridae family (figure 1) (McLaughlin-Drubin and Munger, 2008). It is approximately 55nm in diameter (Zheng and Baker, 2006).

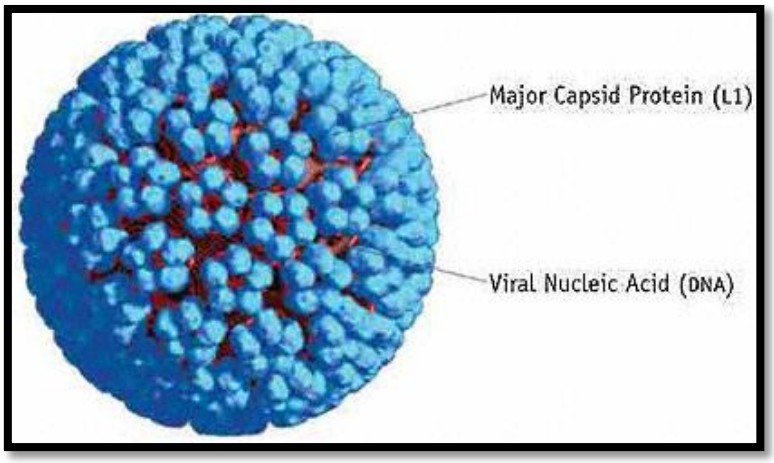

Figure 1: The general structure of HPV (http://removewartsfast.com accessed 20.12.16)

1.1.4.1 Human papilloma virus genomic organization

HPVs are responsible for causing a range of epithelial hyperplastic lesions (Pascale *et al*., 2013). Being epitheliotropic (Gomez and Santos, 2007; Piana *et al*., 2009), HPVs infect the cutaneous and mucosal epithelial tissue of the anogenital tract, hands and feet and are responsible for causing cervical, oral, anogenital types of cancers. Their genomes (figure 2) are approximately 8kb in size (De Villiers *et al*., 2004; WHO, 2015). Currently, there are over 300 different HPV types which have been identified (Chaturvedi *et al*, 2011; Egawa *et al*., 2015). The mucosal and cutaneous types are further divided into low, high risk and potential high risk types (table 1) and this depends on the lesion's property for malignant progression (Oncogenic potential). The HPV genome (figure 1) is organised into three regions; an early, late and a long control region (LCC) (also called upstream regulatory region (URR) the no coding region (NCC)) (Gomez and Santos, 2007; Egawa *et al*., 2015). This LCC represents 10% of the genome (Camilleri and Bundell, 2009). The LCC contains the p97 core promoter along with enhancers and silencer sequences that regulate DNA replication by controlling the transcription of the early and late regions (regulation of gene functions). It also contains the highest degree of variation in the viral genome (Gomez and Santos, 2007).

The early region representing 50% of the HPV genome includes the genes E1, E2, E3, E4, E5, E6, E7, and E8 (Table 2) (Steben and Duarte-Fanco, 2007). This region is involved in viral replication and oncogenesis. The late control genes (representing 40% of the HPV genome) encodes L1 (major capsid protein) and L2 (minor capsid protein) structural proteins of the viral capsid (Villa, 2006; Gomez and Santos, 2007 and Stanley, 2010). The E6 and E7

genes are products of 2 open reading frames from the early control region and are important in the HPV-induced processes of cellular transformation and immortalization. One third of the HPV types affect the genital tract (e.g. genital warts), while other types cause plantar warts (Clifforda *et al*., 2006; Villa, 2006, McLaughlin-Drubin and Munger, 2008).

Table 1: Different types of HPVs (http://i516.photobucket.com)

Association with cervical cancer[1]	Genotypes
Low-risk	• Most common: 6 and 11 • 40, 42, 43, 44, 54, 61, 70, 72, 81 and CP6108
Probable high-risk	• 26, 53 and 66
High-risk	• Most common: 16, 18 • 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82

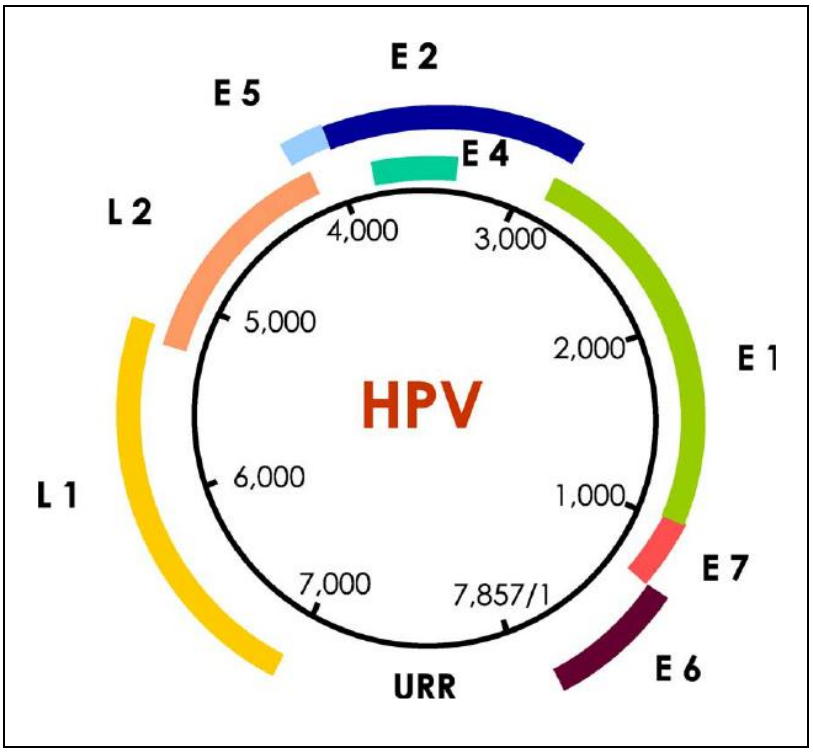

Figure 2: General organisation of the Human Papilloma Virus genome. Source: (http://gallery4share.com accessed on 25.August 2015).

Table 2: Human Papilloma virus gene functions (Gomez and Santos, 2007)

Gene category	Gene	Function
Early genes	E1	Viral replication
	E2	Modulation of transcription and replication
	E3	Unknown
	E4	Productive viral infections
	E5	Transforming properties
	E6	Oncoprotein; interaction with p53 protein
	E7	Oncoprotein; interaction with pRb
	E8	Unknown
Late genes	L1	Major capsid protein
	L2	Minor capsid protein

1.1.4.2 Epidemiology of human papilloma virus

Human Papilloma virus infection is prevalent in both men and women (Garland, 2010) and it accounts for 5.2% of all cancers worldwide (Ma *et al*., 2012). It was estimated that 6.2 million people are infected with HPV every year in the United States of America (Dunne *et al*., 2006). The prevalence of HPV infections may be as high as 64% in South Africa according to Katz *et al*., (2013). It is the most frequently acquired sexually transmitted infection worldwide (Watson, 2005; Gomez and Santos, 2007) and causes more malignancies than any other virus (Trimble, 2012).

1.1.4.3 Human papilloma virus pathology

Skin to skin contact is the primary mode of HPV transmission (Stanley, 2010) and HPV infects the basal cells of stratified squamous epithelium. Other types of cells are moderately resistant to infection by HPV. The HPV replication cycle begins (figure 3) when the virus gains entry into the cells of the basal layer of the epithelium (Peevor and Fiander, 2010; Egawa *et al*., 2015) and this requires a mild abrasion of the epidermis usually caused by a mild trauma (Stanley, 2008). Even though the receptor for entry of the virus into the cell is unknown, heparin sulphanate mediates the initial attachment of the virus into the cell (Longworth and Laimins, 2004; Gomez and Santos, 2007).

The virus enters the cell and uncoats its viral DNA through the disruption of intracapsomeric disulfide bonds; this reduces the environment of the cell which allows viral DNA to be

transported to the nucleus. This is followed by the maintenance of the genome at a low copy number episome (about 50-100 copies per cell) in the basal cells of the epithelium this is known as latent infection (Stanley, 2008; Peevor and Fiander, 2010). The viral proteins E1 and E2 are expressed in order to maintain viral DNA as an episome and to facilitate the correct segregation of genomes during cell division (Doorbar, 2005).The integration of HPV DNA deletes the E2 region which results in the loss of its expression. This interrupts the function of E2, which normally down regulates the transcription of E6 and E7 genes (Stanley, 2010).

During a productive HPV infection, the function of the E6 and E7 genes is to destabilize the cell growth regulatory pathways and modifying the cellular environment in order to aid in viral replication. The E6 and E7 binds and inactivates two suppressor proteins, p53 and retinoblastoma gene product (pRb), thus disregulating the host cell growth cycle (Wright, 2006). E6 binds to p53 resulting in its degradation, the absence of p53 suppressor protein, DNA damages can accumulate without repair. E7 binds to pRb tumour suppressor protein, and these binding results in the disruption of the pRb and E2F-1 (cellular transcription factor) complex, hence liberating E2F-1. The E7 also associates with other interactive cellular proteins such as cyclin E (Longworth and Laimins, 2004).The removal of pRb results in over expression of E2F transcription factor with upregulation of cell cycle genes. This results in DNA replication, transition of the cell from the G1 to the S phase, and in increased cell proliferation. In combination, these factors can promote the production of cells with malignant phenotype and the process can take several years to develop (Schiller *et al*., 2010).

The HPV genome replicates in the nucleus of the infected cells in the form of a closed circular episome that is amplified into high copy numbers (at least 1000 copy numbers) after the differentiation of basal cells into keratinocytes (Stanley, 2008). The infected cell moves towards the epithelial surface, the late genes (L1 and L2) are activated and produce their products. Following virion assembly, mature viruses are released from the uppermost layers of the epithelium to infect other cells. Thus the cycle repeats (Longworth and Laimins, 2004).

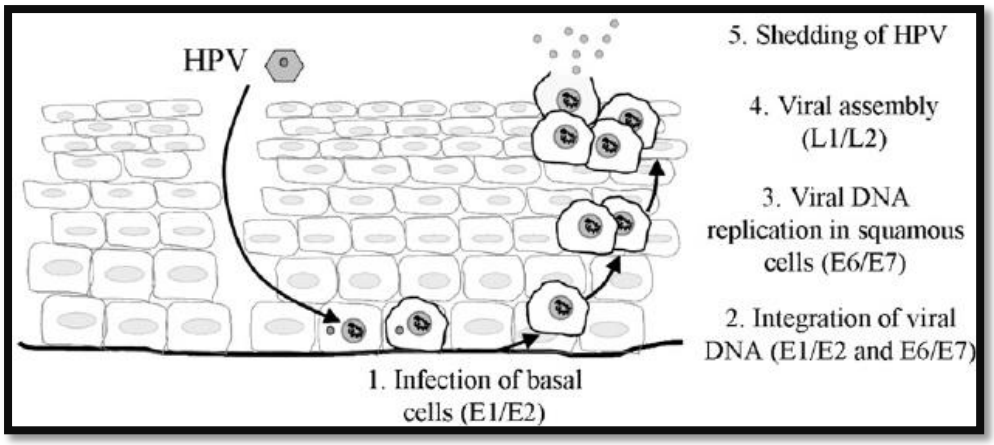

Figure 3: Life cycle of Human papilloma virus (Stanley, 2008).

1.1.4.3 Prevention and treatment of human papilloma virus

The life time risk of acquiring HPV in unvaccinated individuals is 80% (Trimble, 2012). The vaccines that are available in South Africa include Cervarix and Gardasil which were licensed in 2008 (Francis and Mira, 2013; Katz *et al*., 2013; Hoque and Van Hal, 2014) and they have only been accessible to a small portion of the population (20-25%). The remaining 75-80% of the population which is most at risk of HPV infection has no access to the vaccine (Katz *et al*., 2013).These vaccines use type specific L1 proteins that assemble themselves into virus like particles (VLPs). Cervarix is a bivalent vaccine against HPV types 16 and 18 while Gardasil is a quadrivalent vaccine against HPV types 6, 11, 16 and 18 (Feller *et al*., 2010). The L1 protein is expressed through a recombinant baculovirus vector in the bivalent protein. These vaccines function by inducing neutralising serum antibodies (IgG). It's been reported that these vaccines are effective; with 98% and 92% efficacy for Gardasil and Cervarix respectively (Peevor and Fiander, 2010). Since HPV is sexually transmitted, Cervarix and Gardasil should be administered to young girls and boys before they commence sexual activity (Denny, 2009; Feller *et al*., 2010). These vaccines do not offer protection against previously existing HPV infections (Grm *et al*., 2009).

Although these vaccines are effective, they also have some side effects. The common side effects include pain, swelling and erythema at the injection site for patients vaccinated with either vaccine. The side effects specific to Cervarix are headache, fatigue and mylagia; and fever for Gardasil (Peevor and Fiander, 2010). Other forms of treatment for HPV include the use of topical treatment such as Imiquimod cream and Cidofovir. Imiquimod cream is licensed for treatment of anogenital warts, superficial basal carcinomas and actinic keratinoses. Cidofovir is an acyclic nucleoside phosphonate, having a broad spectrum of antiviral activity with in vitro and antitumor potential. It has the ability to decrease E6/E7 gene

expression thus increasing the levels of p53 and Rb; and consequently induce apoptosis of HPV infected cells (Peevor and Fiander, 2010).

1.1.5 Hepatitis B Virus biology

Hepatitis viruses are small enveloped partially double stranded circular DNA viruses belonging to the Herpesviridae family (Welzel *et al*., 2006). They are the smallest human pathogens (Venkatakrishnan and Adam, 2016). The virion is also known as the Dane particle 42nm in diameter. The virus consists of an icosahedral core containing, dsDNA enveloped by a membrane with embedded surface proteins (figure 4) (Short *et al*., 2009).

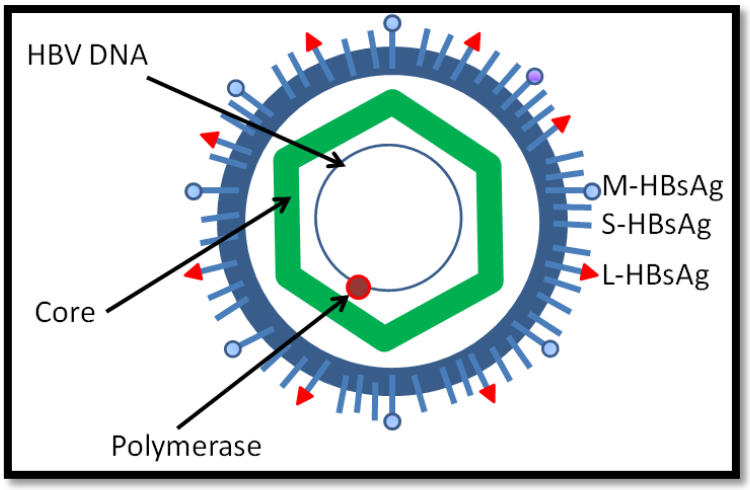

Figure 4: The structure of Hepatitis B virus (https://en.wikipedia.org accesesd on 20.12.2016).

1.1.5.1 Hepatitis B virus genomic organization

HBV is a partially double stranded DNA virus that is classified in the family Hepadnaviridae (Welzel *et al*., 2006). The HBV genome, compact and 3.2 kb in size, is organised into four partially overlapping open reading frames which encode the envelope, icosahedral core (precore/core), polymerase and x proteins (figure 5). The core contains a partially double stranded genome and the envelope proteins are surface glycoproteins designated as hepatitis B surface antigen (HBsAg) (Kradjen *et al*., 2005; Crowther, 2008).

The surface proteins exist in three sizes, namely small (S), medium (M) and large (L) which form disulfide bonded homo and heterodimers. These three proteins share a common C terminal region though they are expressed from different start sites. The M protein contains preS2 sequence N terminal to S, while the L protein has a further preS1 sequence N terminal to M. In virus infected hepatocytes, HBsAg is produced in excess and secreted into blood where it serves as a marker of infection and infectivity (Short *et al*., 2009). The surface

protein genes entirely overlap the viral polymerase gene. The x protein is not well understood but has been implicated in carcinogenesis (Fung *et al*., 2009).

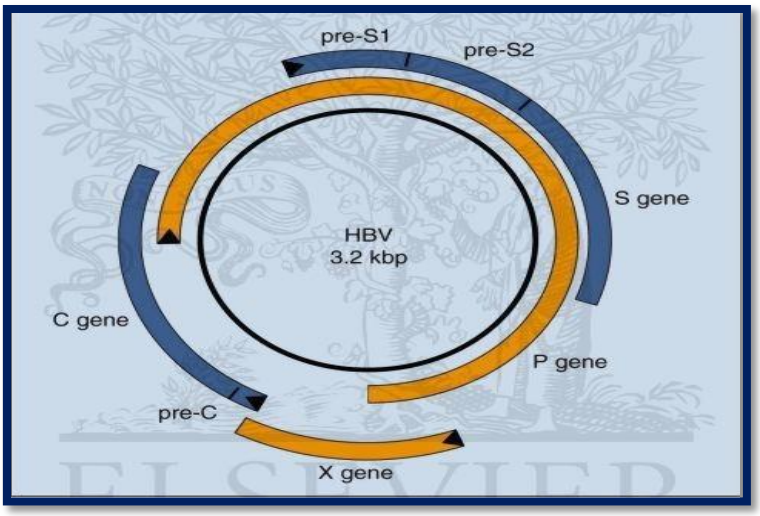

Figure 5: The general organization of the HBV genome. Source: (Elsevierimages.com accessed on 03 August 2015).

1.1.5.2 Epidemiology of HBV

Hepatitis B prevalence varies geographically, with 45% of the global population living in endemic areas (>8% prevalence of the hepatitis B surface antigen (HBsAg) notably the Asian-Pacific and sub-Saharan African region). About 65 million people in Africa are chronically infected; 2.5 million of them reside in South Africa (Spearman and Sonderup, 2014)**.** HBV prevalence in South Africa varies owing to gender, race, age and geographic location of the studied population; however; the general population has a range of 1-10% HBV prevalence (Burnett *et al*., 2005; Mayaphi *et al*., 2012). The prevalence of chronic HBV can be categorised into three categories namely high, intermediate, and low endemicity (Ramezani *et al*., 2011; WHO, 2015).

Hepatitis B is highly endemic in developing areas with large populations which includes South East Asia, China, Sub-Saharan Africa and the Amazon Basin. In these areas, 8% of the population are chronic HBV carriers (Hou *et al*., 2005). HBV is moderately endemic in parts of Eastern and Southern Europe, the Middle East, Japan and part of South America. About 10-60% of the aforementioned populations have evidence of infection and 2-7% are chronic carriers. Most developed areas such as North America, northern and western Europe; and Australia have low HBV endemicty. About 5-7% of the population are infected by HBV in these regions and only 0.5-2% of the population are chronic carriers (Hasan, 2005).

1.1.5.3 HBV genotypes

To date HBV consists of 10 genotypes, namely A through J, with distinctive geographical distribution (table 3) (Sunbul, 2014). These genotypes can be further subdivided into subgenotypes (A1-7), B (B1-6), C (C1-5), D (D1-9), F (F1-4) with exception to genotype E, G and H. Genotype A is subdivided into seven subgenotypes respectively. HBV classification is based on >8% nucleotide differences for genotypes as well as 4-8% nucleotide difference for subgenotypes. These genotypes vary in geographic distribution (Table 2). Genotype A is more prevalent in North America, northen and western Europe and sub-Saharan Africa (Kew, 2008; Mora *et al*., 2010; Sunbul, 2014). These different HBV genotypes and subgenotypes show different clinical and virologic manifestations. As an example genotypes A and B show better responses to alpha interferon therapy than genotypes C and D (Sunbul, 2014).

Table 3: Distribution of HBV genotypes and subgenotypes among countries (Adapted from Sunbul, 2014).

Country	Genotypes	Sub-genotypes
China	B,C	B2, C1, C3
Indonesia	C, B	C1, B3, B7, C10, B9 and C8
Tunisia	D, F	-
Turkey	D	D2, D1, D3
Brazil	A,F	A1, F2a,A2,F4
Vietnam	B, C, I	B2-5, C5-16
Taiwan	B	B2, B3
South Korea	C	-
Hong Kong	C, B	-
Gambia, Nigeria	A	A4, A5, A6, A7
Haiti, Congo, Rwanda, Cameroon	-	-
Japan	A, C	C1, C2, C3
Philippines	A,B, C	A1, B5, C5
India	A, C, D	-
Canada	C,B, A,D	-
Central African Republic	A, D,E	A1, D4
Saudi Arabia	D, E	D1
Iran	D	D1
Mongolia	D	-
South Africa	D	D3
Thailand	C, B	C1-5
Italy	D	-
Morocco	D, A	D1, D7, A2
Argentina	F	F1, F2, F4
Egypt	D	D1
Pakistan	D	-
Australia	C, D	C4, D4
Spain	A, D, F	-

1.1.5.4 Hepatitis B virus pathology

The HBV life cycle has a unique life cycle that results in the production of a large number of viral loads during active replication without actually killing the infected cells directly (Locarnini and Zoulim, 2010). The early stages of HBV life cycle involve attachment, penetration and uncoating (figure 6). The virion binds to the surface receptors of the liver cell with the aid of the interaction of the preS region of the large envelope polypeptide and then enters into the cytoplasm where it uncoats (Locarnini and Zoulim, 2010). The nucleocapsid particle delivers the viral genome to the nucleus where the partially single stranded DNA plus strand is closed.

The 3.2 kb covalently closed circular DNA (CCC DNA) which serves as the transcriptional template of the virus is then formed (Locarnini, 2004). The CCC DNA is a very stable structure which can stay in the host nucleus for months in chronic infection. Transcription driven by polymerase enzyme II results in the production of four viral RNAs that are actively transported out of the nucleus. This occurs through shared sequences at the end of the transcripts that interact with RNA export proteins. Once in the cytoplasm, the transcripts are translated into the respective proteins (Minor and Slagle, 2014).The precore protein contains a leader sequence that transports it into the endoplasmic reticulum where it is further processed as HbeAg (Crowther, 2008). This envelope protein then transverses the ER membrane as an integral membrane protein.

The core and polymerase proteins assemble around the pregenomic RNA (pRNA) to form HBV-RNA containing capsids within which the RNA is reverse transcribed to produce the first strand viral DNA that serves as a template for second strand DNA synthesis (Crowther, 2008).The RNA containing capsid matures into a DNA containing capsid and migrates bidirectionally within the cytoplasm. One pathway terminates at the ER membrane where it interacts with the envelope proteins which initiate internal budding reaction (Chisari, 2000). This results in the production of virions that are transported out of the liver cell through the default secretory pathway. The second pathway transports the capsid to the nucleus to amplify the pool of cccNDA (Chisari, 2000).

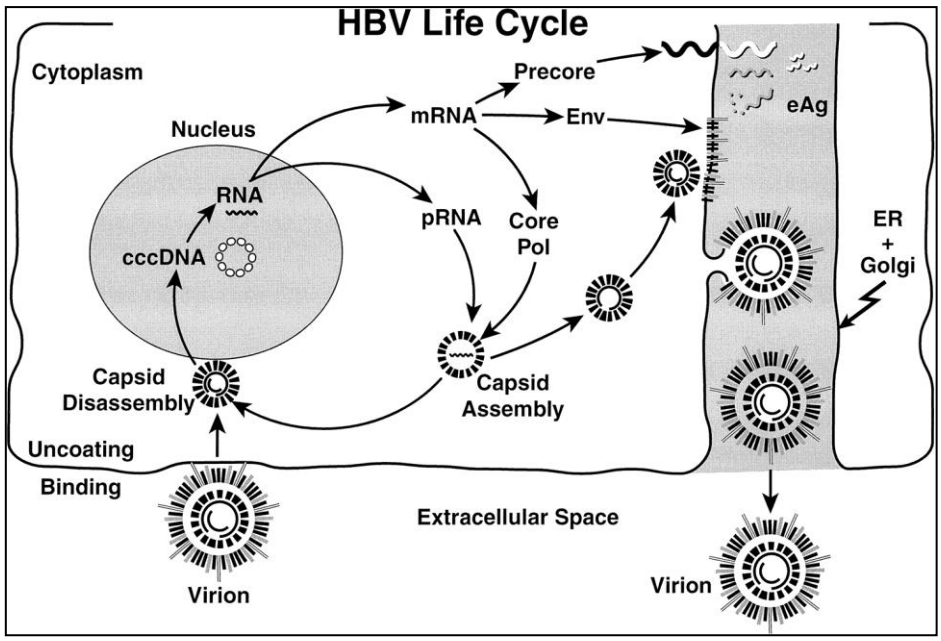

Figure 6: The life cycle of HBV (Chisari, 2000)**.**

1.1.5.5 HBV treatment

The aim of HBV treatment is to get rid of HBV DNA (together with HBsAg and HbeAg) in order to prevent cirrhosis, liver failure as well as hepatocellular carcinoma. Available treatment include nucleoside analogues (NUCs) (such as lamivudine, adefovir, entecavir, tenocavir and telbivudine) and interferon based therapies (conventional and pegylated alpha). NUCs suppress the viral replication by inhibiting HBV viral polymerase and the interferon therapies enhance host immune response (Apinall *et al*., 2011).

1.1.6 Human Herpes Virus type 8 biology

HHV-8 also known as Kaposi's sarcoma associated Herpesvirus (KSHV) is a double stranded DNA virus, which is 120 nm in diameter and 165kb in size (McLaughlin-Drubin and Munger, 2008) (figure 7) and falls under the virus belongs to family Herpesviridae, which is subdivided into alpha, beta and gamma herpesviridae (the first of the genus Rhadinoviruses virus shown to infect humans).

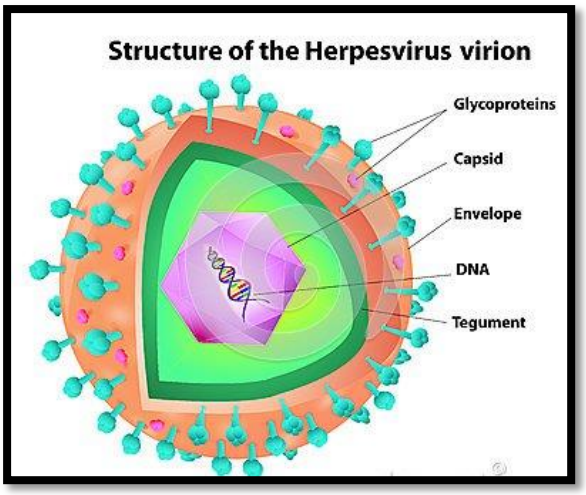

Figure 7: The structure of HHV-8 (dreamstime.com accessed 20.12.16)

1.1.6.1 HHV-8 classification, genomic organization and treatment

HHV-8 also known as Kaposi's sarcoma associated Herpesvirus (KSHV) is a double stranded DNA virus, which is 120 nm in diameter and 165kb in size (McLaughlin-Drubin and Munger. 2008) and falls under the virus belongs to family Herpesviridae, which is subdivided into alpha, beta and gamma herpesvirinae (the first of the genus Rhadinoviruses virus shown to infect humans). The alpha herpesvirinae (herpes virus type 1, 2 and Varicella-zoster virus) have a short replication cycle, induce cytopathology in monolayer cell cultures and have a broad host range. The beta herpesvirinae (cytomegalovirus, human herpes virus 6 and 7 (HHV6, HHV7)) possess a long replication cycle and have a restricted host range. The gamma herpesvirinae which includes EBV and HHV-8; possess a very restricted host range (Edelman, 2005).

Diseases associated with HHV-8 include Kaposi's Sarcoma (KS), primary effusion Lymphoma and Castleman's multicentric disease (Ishak *et al*., 2007). The HHV-8 genome consists of a long unique coding region which encodes over 80 open reading frames (ORFs). These ORFs are surrounded by terminal repeat regions (TRs) consisting of 801 base pair direct repeat units with a high Guanine and Cytosine content. The ORFs are named after the corresponding genes in herpes virus saimiri and the genes without positional homology are numbered with K prefix. The large regions (I, II, III) with unique regions among them contain genes conserved among rhadinoviruses. Those unique genes (shown in red with black dotted outlines) encode homologues for host cellular proteins while those without known homologues are shown in yellow with black dotted outlines (figure 5). The labeled genes are potentially important in the pathogenesis of KS (adapted from http://www.luhs.org/index.htm accessed on 10.07.2015).

1.1.6.2 Epidemiology of HHV-8

Human herpes virus type 8 (HHV-8) is considered a causal agent of Kaposi's sarcoma (KS). HHV-8 is the first human herpes virus identified after the discovery of Epstein Bar virus (EBV) (Bruce *et al*., 2013). KS is classified into four main groups, classical or sporadic KS (CKS), endemic KS (EKS); Acquired Immunodeficiency Syndrome (AIDS) associated KS (AKS) and iatrogenic KS (IKS) (Dittmer and Damania, 2013). CKS is a slow growing indolent tumor developing in males mainly in eastern and Mediterranean Europe; EKS is predominant in eastern and sub-Saharan Africa and similar to CKS, has been seen in children in a more fatal and fulminant form and AKS is the most common aggressive and rapidly growing type of KS in AIDS patients. IKS is commonly found in drug related immunosuppressed patients such as transplant patients emphasizing the significance of immune disturbance as a co-factor in the pathogenesis IKS, AKS (Pyakurel *et al*., 2007).

The worldwide prevalence and risk factors of HHV-8 infection vary geographically across different populations. Infection with HHV-8 in countries such as North America, northern/ western Europe, is rare with about 5% among healthy blood donors. In areas such as Greece, southern Italy, sub-Saharan Africa, where KS is endemic, prevalence of the virus is much higher specifically among populations such as the elderly men. Studies conducted in Africa reported HHV-8 seropositivity between 32% and 100% in adult populations (Klaskala *et al*., 2005), and 32-60% in southern Africa (Isaacs *et al*., 2016). A number of studies in Africa demonstrate that the majority of children develop HHV-8 antibodies between the ages of 1 to 13 years (Mbulaiteye *et al*., 2003, Dedicoat *et al*., 2004; Butler *et al*., 2011; Dow *et al*., 2013). It is suggested that this may be due to the virus being in the continent much longer or that there may be an unknown co factor that increases the efficiency of HHV-8 transmission (Dow *et al*., 2013).

1.1.6.3 HHV-8 genotypes

HHV-8 genotypes can be classified based on the K1 hyper-variable gene and the highly conserved open reading frame 26 gene (figure 8) (Endo *et al*., 2003) with a low genetic variability (1-2% polymorphisms between HHV-8 isolates) (Zong *et al*., 1997). It is a late lytic transcript encoding a minor capsid protein. This was the first HHV-8 gene to be amplified (Chang *et al*., 1994). Studies done on this gene have resulted in the identification of eight distinctive subtypes namely A/C, J, K/M, D/E, B, Q, R, and N. These subtypes are diversely distributed in different geographical locations. Subtypes R predominates in Africa while K predominates in Greece respectively. Subtypes D/E has been found mostly in South Asian and Polynesia (Pacific Rim) populations, while A/C, J and K have been exclusively identified in almost Eurasian subjects (European, United States, North Asian and Middle Eastern)

(Tornessello *et al*., 2010). Genotypes have also been identified based on the highly variable K1 gene. Among them are genotypes A to E. They are further classified as subgenotypes; A (A1-5), B (B1-4), C (C1-6), D (1-3) and E (E) (Treurnicht *et al*., 2002; Slazai *et al*., 2005; Betsem *et al*., 2014). Subtype A and C occur mainly in Europe, while subtype C predominates in Asia. Subtype B is more common in Africa, subtype D occurs in the Pacific Islands, Asia and the Australia. Subtype E which differs from the all other subtypes by 25-30% has been reported from Brazil (Slazai *et al*., 2005).

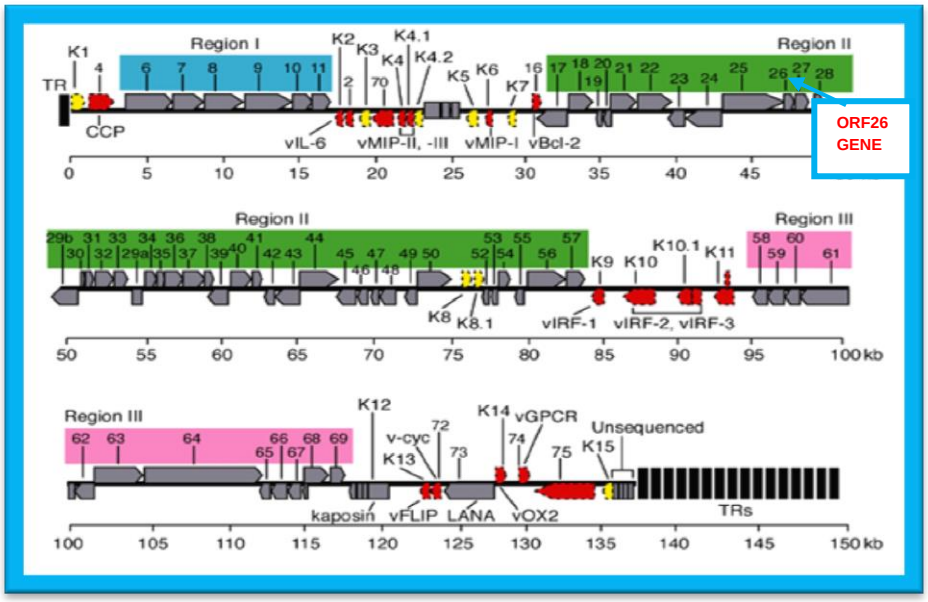

Figure 8: The genomic structure of HHV-8.Source: (Adapted from Neipel *et al*., 1999).

1.1.6.4 Human herpes virus type 8 pathology and treatment

HHV-8 targets mainly the B cells as well as monocytes, endothelial or spindle cells and keratinocytes. HHV-8 completes its life cycle with the latent and lytic phase of infection. The latent state has only a few viral genes expressed (e.g. the viral latency gene) and no infectious progeny is produced. These genes come from the four ORFs including v-FLIP, v-cyclin, as well Kaposin A, B, and C. The lytic state has most viral genes expressed, viral DNA is amplified and infectious virions are released (Liang *et al*., 2003; Ye *et al*., 2011).

Following infection, HHV-8 is endocytosed and remains in the latent state where the virus establishes a lifelong infection. These genes promote survival according to Polson *et al*., 2002. Latency associated nuclear antigen (LANA) suppresses the viral genes needed for viral production and assembly, thus sustaining the latency state. In other words, latency allows the virus to escape immune surveillance and establish persistent infection (West and Wood, 2003). LANA interacts with various cellular proteins, it is capable of binding and

inhibiting the two key cellular tumour suppressing proteins, p53 and Rb. Consequentially, the infected cells become protected from apoptosis and may readily undergo uncontrolled proliferation under certain conditions such as immunodeficiency and immunosuppression. In the latent state, the virus remains as a naked piece of circular DNA (episome) within the infected cells. Upon induction or reactivation (e.g. by immunosuppression), the latent switches to the lytic life cycle with the aid of viral gene (ORF50). Its product, the replication and transcription factor (RTA), is both necessary and sufficient enough to trigger lytic reactivation (Liang *et al*., 2003). The genes expressed during the lytic state include v-IL6, vIRF-1, vIRF-3, K1 and K15 (Cousins *et al*., 2014).

Kaposi's sarcoma is a localized tumor that can be treated by surgery through irradiation. Chemotherapy in combination with drugs such as liposomal anthracylins or paciitavel can be used for invasive disease. The development of KSHV has been successfully prevented by the use of antiviral drugs such as ganciclovir and cidofovir which target HHV-8 replication (Martin *et al*., 1999). Nucleoside analogues such as acyclovir, famcyclovir and valacyclovir can also be used, they act against the HHV-8 lytic phase (Sharma *et al*., 2012).

1.1.7 Prostate carcinogenesis

Prostate carcinogenesis initiation is aided by multiple cancer associated genes that contribute to this process when their functions are disturbed either genetically or epigenetically. The more traditional classes of tumor suppressors (contributes to cancer formation when inactivated) and oncogenes (pro-carcinogenic when activated) in combination with the mutator genes results in a decreased ability to maintain fidelity of the genetic code and function (for example genes involved in DNA repair) when altered (Wang *et al*., 1997; Isaacs and Kainu, 2001).

Human carcinogenesis is a complex process which requires several steps. Evidence for this multistep process was demonstrated in the studies of experimental carcinogenesis in rodent models. A study done by Thompson *et al*., (1989) showed that the expression of a single potent oncogene (for example RAS) in normal prostate cells of the mouse is insufficient for transformation. The over-expression of a second oncogene (myc) is necessary before transformation becomes a frequent event. Even when expressing two oncogenes, not every cell becomes transformed, suggesting that further steps are necessary. These include inactivation of tumor suppressor genes and other growth regulatory elements. Although in PCA clinical specimens the requirement for multiple steps is less demonstrated, the finding of multiple genetic alterations as a common characteristic of prostate cancer and human tumors in general, supports this concept (Isaacs and Kainu, 2001).

Table 4: Candidate regulatory genes for prostate development and carcinogenesis (Shen and Abate-Shen, 2000)

Gene	Product	Mouse and human phenotype
Normal development		
Androgen receptor	Nuclear hormone receptor	Required in mesenchyme for initial formation of prostatic buds, and subsequently in epithelium for secretory protein production
Nkx3.1	Homeodomain transcription factor	Expressed in prostatic regions of urogenital sinus epithelium and in newly formed prostatic buds; required for normal ductal morphogenesis and production of secretory proteins
Shh	Secreted signaling factor	Expressed in urogenital sinus epithelium, anti-*Shh* antibodies inhibit prostate morphogenesis
BMP-4	Secreted member of TGFβ superfamily	Defective prostate morphogenesis in heterozygous mice
FGF7	Growth factor	Stimulates prostatic growth in culture; mutant mice do not display prostatic defects
FGF10	Growth factor	Expression in prostate is androgen-regulated; stimulates growth of prostate epithelium
TGFβ1	Growth factor	Implicated as a regulator of androgen signaling; mutant mice display defects in prostatic duct formation
HoxD13	Homeodomain transcription factor	Expressed in the developing and adult prostate; mild defects in prostatic morphogenesis in mutant mice
Initiation and progression to carcinoma		
NKX3.1	Homeodomain transcription factor	Prostatic epithelial hyperplasia and dysplasia followed by PIN in aged heterozygous and homozygous mutant mice; prostate-specific expression in human and mouse adult tissues; human gene maps to minimal deleted region of 8p21, but not mutated in human tumors
PTEN	Lipid phosphatase	Heterozygous mutant mice develop hyperplasia and dysplasia of multiple tissues including prostate; human gene maps to 10q23, but status of mutations is unresolved
MXI1	Transcription factor	Relatively mild prostatic epithelial hyperplasia and dysplasia in homozygous mutant mice; human gene maps to 10q24, but is infrequently mutated
Rb	Cell-cycle regulator	Homozygous mutant mice prone to hyperplasia, dysplasia and carcinoma in combined prostatic rescue and hormone induction model; human gene maps to 13q and functional studies suggest a critical role, but it is infrequently mutated
p27	Cell-cycle regulator	Homozygous mutant mice develop hyperplasia and dysplasia of multiple tissues including prostate; loss of expression in human tumors correlates with tumor grade
p16	Cell-cycle regulator	Protein expression is up-regulated in carcinoma, but mutations are infrequent; limited information is currently available on the prostate phenotype of mutant mice or on the status of other *INK* family members

(Continued)

Gene	Product	Mouse and human phenotype
Telomerase	Ribonucleoprotein	Reduced telomere length and increased telomerase activity found in PIN and carcinoma
Myc	Transcription factor	Amplified in some carcinomas; cooperates with *RAS* to induce hyperplasia in tissue recombinants
FGFs	Growth factors	Several family members, including *FGF7* and *FGF10*, are implicated as regulators of prostatic growth; altered FGF function associated with progression in TRAMP mice
E-cadherin	Cell adhesion	Reduced expression in PIN and carcinoma; loss may be associated with poor prognosis
c-CAM	Cell adhesion	Expression is reduced in PIN and lost in carcinoma
Integrins	Cell interactions	Reduced expression of specific family members during cancer progression
c-Met	Tyrosine-kinase receptor	Overexpressed in PIN, carcinoma, and metastasis
Advanced carcinoma and metastasis		
Androgen receptor	Nuclear hormone receptor	Expression maintained even in androgen-independent tumors, although it is often amplified or mutated
p53	Transcription/ apoptotic regulator	Mutation rate in is low in primary cancer; frequently mutated in metastasis; *p53* overexpression correlated with poor prognosis
Bcl2	Apoptotic regulator	Overexpression confers resistance to apoptosis in androgen-independent disease; key target for clinical intervention
IGF1	Growth factor	Promotes growth of prostate epithelium; elevated serum levels associated with cancer risk; overexpression of *IGF1* in TRAMP mice associated with progression
TGFβ1	Growth factor	Negative regulator of prostate growth; shift to autocrine regulation associated with metastasis
EGF/TGFα	Growth factor	Stimulates prostatic epithelial cell growth and invasiveness; may provide a mechanism for overcoming androgen-dependence
Ka1	Putative integral membrane protein	Shown to suppress metastases; protein expression is down-regulated but is not mutated

1.1.8 Viral oncogenic mechanisms

Viruses associated with human cancer are known as tumor viruses; most of which are capable of integrating into the host genome and have the ability to immortalize the target cell in order to allow their own replication. The infected gene expresses the viral genes, which are able to induce cell growth, proliferation and prevent apoptosis (Carrilo-Infante *et al*., 2007).

Tumor viruses can be classified as either RNA or DNA tumor viruses based on the type of nucleic acid material packed in the infectious viral particle (Bergonzini *et al*., 2010) (table 5). DNA tumor viruses (e.g. adenoviruses, papilloma viruses) cause cell transformation by exclusively encoding proteins of viral origin which are vital for replication (Carrilo-Infante *et al*., 2007). The DNA tumor viruses' oncogenes lack identifiable sequence similarities to cellular genes. The ability of these viral genes to transform cells was elucidated during the late 1970s. According to Martin *et al*., 1975, it was demonstrated that SV40 was capable of inducing tumor formation in experimentally infected hamsters by viral tumor (T) antigen expression, which is the major oncogenic determinant of SV40.

Table 5: Human oncogenic viruses (Zheng *et al*., 2010)

Taxonomic Grouping	Examples	Oncogenes	Tumor Types
1. DNA viruses			
Adenoviridae	Adenovirus types 12, 18, 31	E1A, E1B	Various solid tumors only in rodents
Hepadnaviridae	HBV	HBx	Hepatocellular carcinoma
Herpesviridae	EBV	LMP-1, BARF-1	Burkitt's lymphoma, B-cell lymphoma, NPC
	KSHV	vGPCR	Kaposi sarcoma, primary effusion lymphoma
Papovaviridae	Merkel cell polyomavirus	T antigens	Merkel cell carcinoma
	BK virus, JC virus		Solid tumors in rodents and primates
Papillomaviridae	HPV 16, 18, 31, 45	E6, E7	Cervical and anal cancer, Oral cancer
2. RNA Viruses			
Flaviviridae			
Hepacivirus	Hepatitis C virus	?	Hepatocellular carcinoma
Retroviridae			
HTLV	Human T-cell leukemia virus type I	Tax	Adult T-cell leukemia/lymphoma

The finding that the SV40 large T antigen interacts with a cellular protein with a low molecular weight 53 KDa resulted in naming the cellular protein as p53 based on its size. This finding was the first evidence that products of DNA tumor virus oncogenes could function as physical or direct interactions with the cellular proteins. As a result of cloning p53 genes from neoplastic rodent and human cells, it was possible to demonstrate that in all cases coding sequences differed from those present in normal cells by carrying important gain of function mutations (Bergonzini *et al*., 2010). In almost 50% of all human cancer cases worldwide, the p53 gene is mutated or lost. This represents the most commonly mutated gene in human tumors. This finding suggests p53 to act as a tumour suppressor gene, which in contrast to proto oncogenes (a normal gene that can become an oncogene by a small modification of its function such as loss of gene regulation) function to promote cancer (Braithwaite and Prives, 2006).

1.1.9. The P53 pathway

The accumulation and activation of the p53 protein is achieved by a wide variety of stress signaling stimuli that engage the p53 network. These include DNA damage and viral infection, although hypoxia may also be significant. These stress signaling stimuli induce p53

activation which binds to and regulate the activity of several important cellular factors (Braithwaite and Prives, 2006). Once activated, p53 can function as a sequence specific transcription factor that can directly act to turn on and off various genes that affect cell growth and survival. Specifically, the activated p53 gene regulates cell cycle progression, senescence, DNA repair, recombination and apoptosis thus preventing tumor formation by reducing the accumulation of genetic lesions (figure 9). In this manner p53 can prevent tumor formation by reducing the likelihood with which genetic lesions that may contribute to tumorigenesis accumulate. When p53 function is compromised or inactivated, the cells are often predisposed to tumor formation (Bergonzini *et al*., 2010). In the case of viral infection, p53 activation means an attempt of the host cell to inhibit viral replication, by inducing, for example, apoptosis. As a result a number of DNA viruses have evolved proteins such as the SV40 large T antigen, to bind and activate p53 in order to bypass cellular antiviral response with a consequence of cell transformation (Butel, 2000).

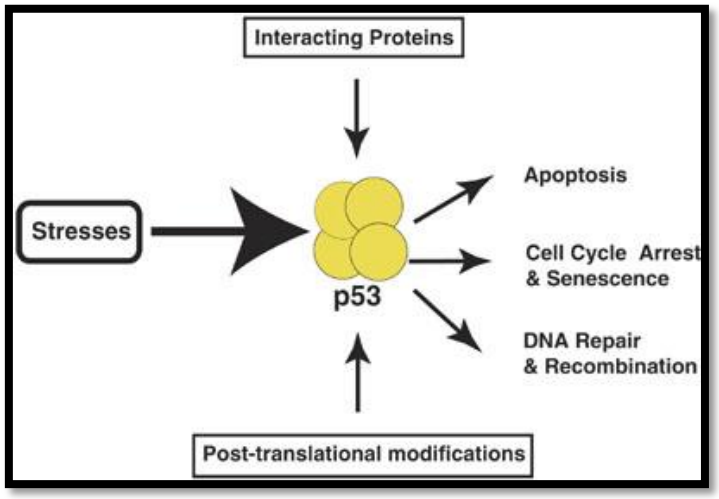

Figure 9: A simplified cartoon of p53 activation and response to stress stimuli (Braithwaite and Prives, 2006).

Another mechanism which DNA oncogenic viruses use to induce tumors exists. This mechanism involves a second tumor suppressor gene called retinoblastoma (Rb), discovered through childhood tumour retinoblastoma. Rb susceptibility was linked to a recessive trait and the gene encoding a specific tumor suppressor gene was identified and cloned and the protein named Rb (Friend *et al*., 1986). The RB protein immunoprecipitates with adenovirus EIA and with SV40 large T protein from transformed cells. The EIA and SV40 large T antigen with Rb was essential for understanding the cellular tumor suppressor function with the demonstration that a phosphorylated form of Rb negatively regulates G1 to S phase progression (Giacinti and Giordano, 2006). This occurs through the cell cycle by binding to and inhibiting the activity of E2F, which is also a transcriptional factor activating several genes involved in cellular DNA replication. Cell progression through G1 to S phase

depends on the G1 cyclin kinase dependent activity which directly hyperphosphorylates and inactivate Rb leading to the release of active E2. Viral oncoproteins specifically bind to and inactivate the hyperphosphorylated form of Rb. This results in the free accumulation of E2F, with consequent uncontrolled cellular proliferation.

The p53 and Rb proteins are two of the most important cellular tumor suppressor proteins. A common theme of DNA tumor viruses emerged since it has been demonstrated that oncoproteins encoded by SV40, adenovirus and HPV share similar capacities for inactivating both Rb and p53 tumor suppressors. Up to date, the DNA viruses that are constantly associated with human tumors include HBV, EBV, HPV, HHV-8 (Damania, 2007 and Oliveira and Antonio, 2007). The list of cellular or viral protein interactions involved in DNA virus related oncogenic transformation is shown in figure 10.

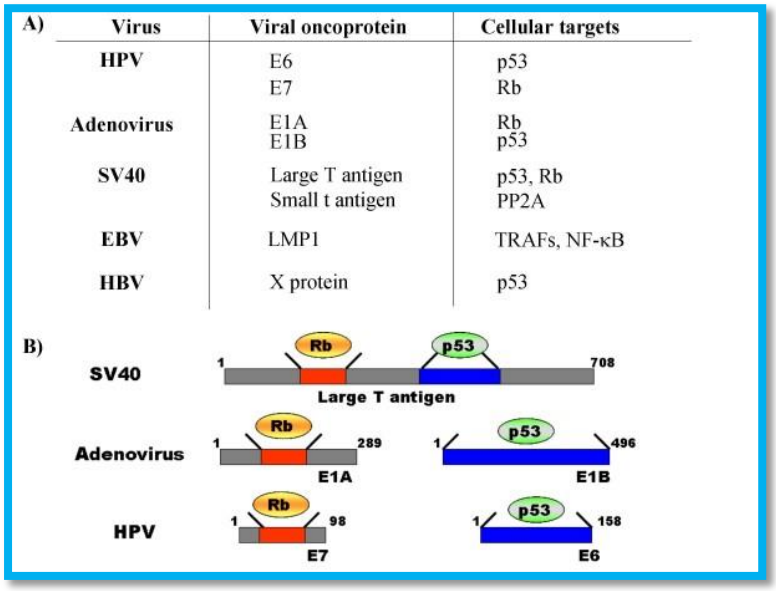

Figure 10: (A) Representative list of cellular or viral protein interactions involved in DNA virus related oncogenic transformation; (B) P53 and Rb are central targets for oncoprotein (Bergonzini *et al*., 2010).

1.1.10 HPV and its association with prostate cancer

HPV represents a typical human oncogenic DNA virus. Specific genotypes (HPV type 16/18) are linked to different forms of cancers, mainly cervical cancer, and penile and upper aero-digestive tract carcinomas (Bergonzini *et al*., 2010). High risk HPVs are the causative agents of cervical cancer. One aspect of infection by these HPVs is that it integrates its viral genome into the cell genome (Szostek *et al*., 2009).

The two principal viral oncoproteins involved in cervical cancer are early genes E6 and E7. These genes have the ability to transform and immortalize primary keratinocytes. These two

oncogenes work by destabilizing tumor suppressor proteins p53 and Rb (Narisawa-Sait and Kiyono, 2007). When tumor-suppressor genes are inactivated, the E6 proteins lead to the degradation of p53 protein and the binding complex of E7 and pRb proteins disturbs the normal cell cycle thus leading to cellular proliferation according to zur Hausen, 2002. A number of studies have been done that show HPV to be somehow associated with prostate cancer.

A study done by McNicol and Dodd, (1990) showed the presence of oncogenic HPV types in prostate tissues, however, a potential of the virus aetiology remains to be demonstrated. A positive association was found between HPV and prostate cancer by Martinez-Feirro *et al*., 2010. The increased prevalence of HPV detected is in agreement with that reported in previous studies, all supporting the association of HPV infection and prostate cancer (Michopoulou *et al*., 2014).

1.1.11 HBV and its association with prostate cancer

HBV is an oncogenic virus that is responsible for the development of cancers such as hepatocellular carcinoma (HCC) (Fung *et al*., 2009). The relationship between HBV and PCA is not well understood and there is limited data on this regard. HBV causes HCC in two pathways, either directly or indirectly (figure 11). Directly, HBV causes HCC by integrating its genome into the host genome. HBV integration can have several mutagenic consequences, this include inverted duplications, amplifications, deletions and translocations resulting in chromosomal instability. Malignant transformation occurs when these genetic alterations confer a selective growth advantage to the affected cell. HBV integration can occur in genes responsible for the control of cell proliferation and differentiation. These genes include the human telomerase gene (HTERT) (which regulates cellular immortalization), MAPK1 and the cyclin (a gene which regulates cellular proliferation) as well as the gene for tumour necrosis factor receptor associated protein 1 (which regulates cellular viability). HBx gene is one oncogene that has generated most interest in HBV related carcinogenesis and is the most commonly integrated gene. The exact mechanism by which HBx induce HCC development is not fully understood (Fung *et al*., 2009).

HBx interferes with hepatocyte's DNA repair system and the controlling elements of the DNA cellular proliferation. In addition, HBx can bind with p53, with subsequent inhibition of p53 mediated apoptosis. Besides HBx, there are other gene elements integrating into the host genome, and they include preS2/S gene which encodes a group of regulatory proteins as well as the envelope proteins (LHBs, MHBs and SBs). Overproduction of these envelope proteins may lead to accumulation in the cytoplasm of the hepatocytes, causing the histological appearance of ground glass hepatocytes. Thus this causes cellular stress with

predisposition of the cells to undergo malignant transformation. Another HBV protein is the HBV spliced protein expressed in chronic hepatitis infection. This protein induces apoptosis and modulates signaling through the transforming growth factor pathway, thus presents a novel pathway of promoting fibrosis and hepatocarcinogenesis (Fung *et al*., 2009).

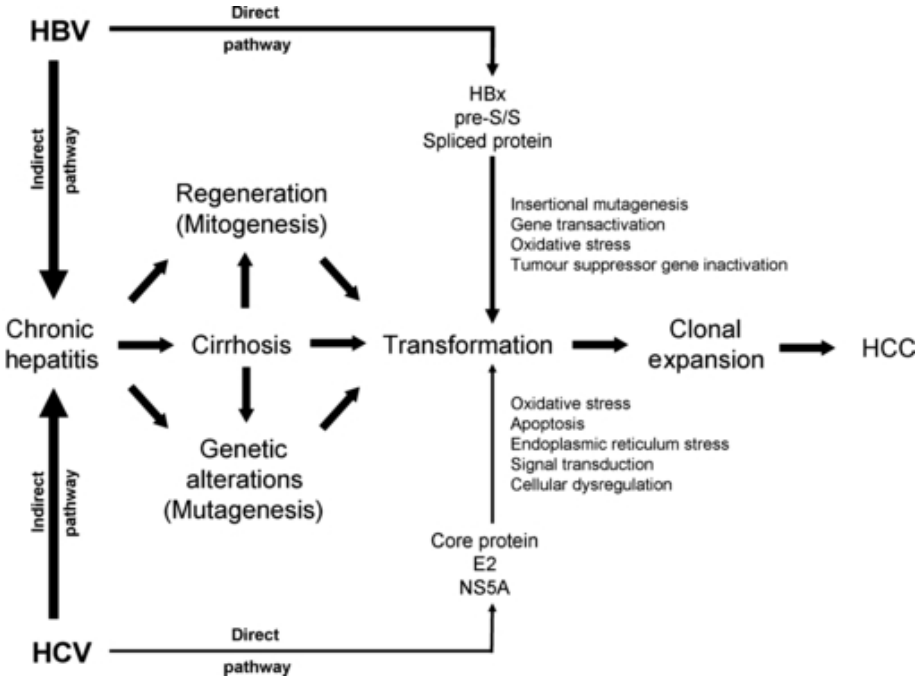

Figure 11: Proposed mechanism of hepatocellular carcinogenesis related to HBV and HCC (Fung *et al*., 2009)

1.1.12 HHV-8 and its association with prostate cancer

HHV-8 represents an example of an inheritably persistent pathogen whose presence in the prostatic reservoir could modify the overall outcome of the disease towards an androgen independent phenotype in high risk individuals. The HHV-8 genome consists of several open reading frames in addition to microRNA's known to change growth properties of infected cells through inhibition of apoptosis, immune surveillance modulation, cell transformation and activation of various signaling pathways relevant to cancer histogenesis (Skalsky *et al*., 2007). HHV-8 targets important signaling pathways involved in oncogenesis. This includes the β-catenin pathway that plays an essential role in the control of cell adhesion and tissue morphogenesis. Due to the finding that the largest tegument protein of Human Herpes Viruses 1, UL36 contains activity, it has also been reported that all members of the Herpesviridae family such as EBV and HHV-8, encode UL36 homologues. This suggests an important role in the viral pathogenesis. In all associated HHV-8 tumors, the presence of the viral LANA correlates with β-catenin over expression (Bergozini *et al*., 2010). HHV-8 lytic genes play an important role in cancer development and progression (Bergozini *et al*., 2010). LANA is a latent protein which is regularly present in all HHV-8 associated diseases and is

important for cellular gene expression regulation as well as viral genome maintenance. It interacts with tumour suppressor genes such as p53 and pRb. This suggests that LANA plays a role in promoting oncogenesis (figure 12) (Jha *et al*., 2016). The relationship between HHV-8 and prostate cancer is not well understood.

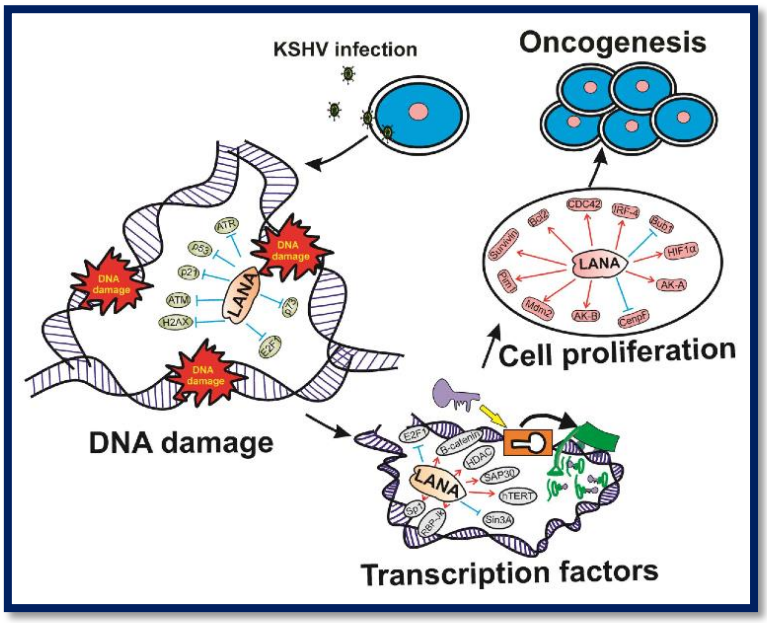

Figure 12: Schematic shows the vital role of KSHV latency associated nuclear antigen (LANA) cancer progression by deregulating DNA damage response, transcription factor activities and cell proliferation properties (Jha *et al*., 2016).

1.1.13 Study rationale

The burden of viral infections in cancer is high and often underappreciated by the research community. It has been estimated by the International Agency for Research that one in every five cancer cases globally are caused by infections, most of them being viruses (Moore and Chang, 2010). The incidence of PCA in Southern African men is 41 per 100 000 of the population per year with a mortality rate of 23 per 100 000 per year (Mofolo *et al*., 2015). About one in every 23 Southern African men will develop PCA during his life time. Although data on prostate cancer in Africa is scarce, it has been estimated by the International Research Agency on Cancer (GLOBCAN) that 57 000 deaths will occur in Africa from PCA by 2030 (Babb *et al*., 2014).

Studies show that approximately seven human viruses (Epstein Barr virus (EBV), hepatitis B virus (HBV); Hepatitis C virus (HCV); Human papilloma virus (HPV); human T cell lymphotropic virus (HTLV-1); Merkell cell polyoma virus (MCPyV) and Kaposi's associated sarcoma virus (KSHV)) are tumorigenic in humans (Schafer *et al*., 2015). These viruses have been found to contribute to about 10 to 15% of the cancers globally (Bergonzini *et al*.,

2010). Cancers caused by viruses that are predominant in Africa in contrast to other parts of the world include cervical cancer (HPV); hepatocellular carcinoma (HBV and HCV); human Kaposi's sarcoma (HHV-8); nasopharyngeal cancer and Burkitt's lymphoma EBV) and Markel cell skin cancer (MCV). These cancers are public health problems in the developing world and the immunosuppressed populations in affluent countries (Sarid and Gao, 2011). The prostate gland has been found to be a habitat for multiple viral infections with oncogenic potential such as HPV, HBV and HHV-8 according to Zambrano *et al*., 2002. Infections from human papillomavirus, hepatitis B virus, and human herpes virus type 8, etiologic agents of different types of cancer are endemic in South Africa. It is therefore important to identify such viruses and understand their mode of action in PCA development as such knowledge is essential in the development of effective vaccines for the prevention of PCA (Martin *et al*., 2009).

In a country such as South Africa, where little is known about PCA (Mofolo *et al*., 2015), more data is required on the possible etiological agents of PCA. South African studies on PCA focus on different aspects of PCA, excluding HPV, HHV-8 and HBV as possible aetiological agents specifically in the Limpopo Province. This study aims to investigate the association of these viruses HPV, HBV and HHV-8 with PCA in South African men.

1.1.14 Research question

- Is prostate cancer associated with Human Papilloma Virus, Hepatitis B Virus and Human Herpes Virus 8 infection?

1.1.15 Hypothesis

- Prostate cancer development is associated with infection with oncoviruses such as Human Papilloma Virus, Hepatitis B Virus and Human Herpes Virus 8.

1.1.16 Study Setting

This is a collaborative study under the auspices of the Southern African Prostate Cancer Study (SAPCS) which addresses different aspects of prostate cancer occurring in South Africa. The SAPCS was established in 2008 (Tindall *et al*., 2014) as a unique resource of epidemiological, genetic and clinical data, as well as associated biospecimens for over 900 South African men with and without prostate cancer. This study is shedding light on the scope and impact of prostate cancer within South Africa. The recruiting hospitals include Tshilidzini, Polokwane, Dr. George Mukhari, and Steve Biko Academic Hospital (figure13).The collaborators in this study include Professors Riana Bornman (University of

Pretoria), Vanessa Hayes (Garvin Institute of Australia), Don Cowan (University of Pretoria) and Pascal Obong Bessong (University of Venda).

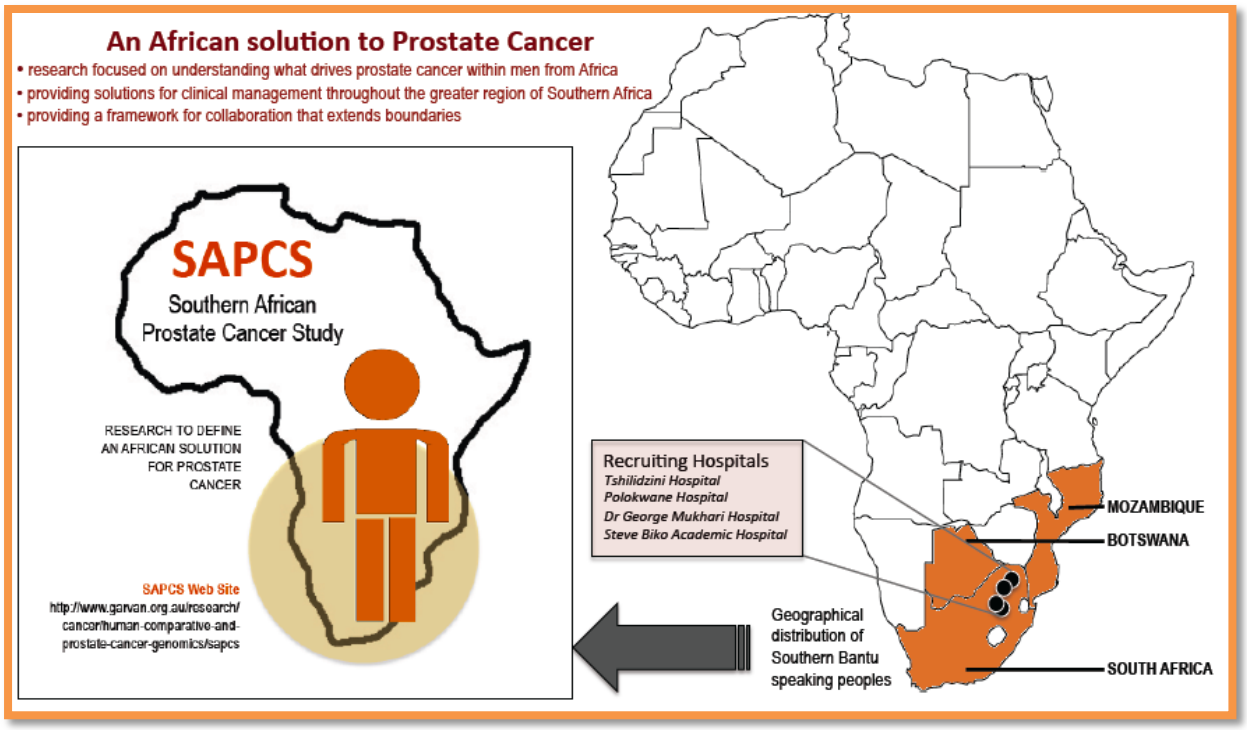

Figure 13: An African map showing the different recruiting hospitals and focus of the Southern African Prostate Cancer Study. Source: (from http//www.spcs.org accessed on 10.09. 2015).

1.16.1 Aims of the SAPCS:

The principal aim of the SAPCS was to provide predictive tools for early detection and disease management, while in addition providing appropriate approaches for the development of much needed treatments.

The first specific aims of the collaborative study is to identify risk factors (specifically diet and chemical exposure) impacting prostate cancer disease and occurrence and adverse outcomes; to identify and validate pathogenic drivers (bacterial or viral) of prostate cancer risk and disease course and to identify and validate biologically relevant DNA drivers (inherited or somatic) of prostate cancer risk and disease course within South Africa.

The current study will focus on identification of viral pathogens in plasma of prostate PCA and BPH patients.

1.1.17 Study objectives

1.1.17.1 General objective

To identify HPV, HBV and HHV-8 in prostate cancer and BPH patients in the Limpopo Province of South Africa.

1.1.17.2 Specific objectives:

1. To determine the prevalence of Hepatitis B surface antigen, Human Papilloma Virus IgG antibodies, and HHV-8 IgG antibodies in males with prostate cancer and BPH.

2. To determine the presence of HBV and HHV-8 DNA in plasma of prostate cancer patients as well as their genotypes through phylogenetic analysis.

3. To determine the associations between HPV, HBV, HHV-8 and PCA.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Ethical consideration

Ethical approval was obtained from the University of Venda's Health, Safety and Research Ethics Committee (SMNS/16/MBY/06). Permission was also obtained from the Limpopo Provincial Department of Health to collect blood samples from both the prostate cancer and BPH patients attending Polokwane Hospital in order to conduct this study. The confidentiality of the patients was protected by replacing identifiable data with codes.

2.2 Study area and population

Polokwane Hospital (also called Pietersburg) is based in Capricorn District Municipality in the Limpopo province of South Africa. Limpopo is home to approximately 5 799 090 people (http://cs2016.statssa.gov.za). The population of the area is 99% African, with 45.9% northern Sotho speakers. The population is dominated by Sesotho speakers with exception to the Afrikaans, English and Tshivenda language speakers. It attracts skilled professionals since it houses a leading regional hospital, Polokwane Hospital (located on the coordinates 23° 53′ 42.06" S; 29° 27′ 44.01" E of the equator). Pietersburg Hospital together with Mankweng Campus Hospital campus is known as Polokwane/ Mankweng Hospital Campus (PMHC). PMHC aims to provide tertiary services to all Level 1 (District) and Level 2 (Regional) hospitals in the Limpopo Province. The hospital gives care to patients from different ethnic groups such as the Northen Sotho, English and Venda speakers.

The study population was a cohort of PCA and BPH patients from South Africa, in the Limpopo province (Polokwane Hospital). The cohort comprised of males and their ages ranged from 49-107 years. A total of 187 histologically diagnosed cancer and benign prostatic hyperplasia (BPH) samples from plasma were used. Clinical and demographic data (obtained from questionnaires and clinical records) of the patients provided included age of diagnosis, stage of the PCA, prostate specific antigen (PSA) level and Gleason score.

2.3 Sample collection and processing

Five milliliter of blood was collected from each consenting individual from South Africa using vacutainer tubes (containing 2x EDTA and others containing 1x Citrate as anti-coagulants) and stored at -80^{o}C. The blood was then centrifuged for five minutes at 4000 rpm. Following centrifugation, plasma was immediately aseptically aspirated and aliquoted into sterile cryotubes and labeled accordingly. The plasma was then stored at -80^{o}C until use.

2.4 Enzyme-linked Immunosorbent Assay (ELISA)

2.4.1 MP Diagnostics HBsAg ELISA 4.1

The MP Diagnostics HBsAg 4.1 kit, (Singapore), was used to screen 187 plasma samples for HBsAg following the manufacturer's instructions. This kit uses antibody sandwich ELISA method in which polystyrene microwell strips are precoated with monoclonal antibodies which are specific for HBsAg. Each patient's plasma sample is added to the wells. This protocol involved three incubation steps. The first incubation step involved the formation of an immunocomplex (antiHBs-HBsAg) due to the presence of HBsAg in the sample which was captured in the solid phase. The second incubation step involved the binding of horse radish peroxidase (HRP) antibodies to the antiHBs-HBsAg immunocomplex formed in the first incubation step, forming an antibody-antigen-antibody complex. A washing step follows which removes any antibodies that are non-specifically unbound. In the third incubation step, a chromogen solution containing trimethyl-benzidine (TMB) and urea peroxidase was added which was converted by the enzyme to a fluorescent signal indicated by blue color formation. A stop solution (Sulphuric acid) was added which converted the blue color to yellow indicating a stop in the reaction.

The intensity of the blue color represents the amount of antigen captured in the wells. Wells containing samples negative for HBsAg remain colourless. The negative and positive controls were checked to validate the results. The optical density was then read according to the test instructions using a microplate reader (VersaMax; Molecular Devices, Silicon Valley, CA, USA). The cut off value turns the measured optical densities into reactive or nonreactive results. The seropositive samples were used for detection of DNA; the seronegative samples were used for occult infection detection. Distribution of HBsAg was stratified according to age, PSA level, and Gleason scores. Significant differences ($p<0.05$) were noted using Chi-square test.

2.4.2 KSHV/HHV-8 IgG antibody ELISA (ABI)

The KSHV/HHV8-IgG antibody ELISA Kit was used to screen 123 plasma samples for KSHV/HHV8-IgG antibodies following the manufacturer's instructions. It was an indirect ELISA kit used to detect HHV8-IgG antibodies to lytic antigens in human plasma. It contains plate wells which are coated with the KSHV/HHV-8 whole virus extract. This test is composed of three incubation steps. The first incubation step involved the addition of the plasma samples into the plate wells where the antibodies specific to KSV/HHV-8 bound to the coated plates, thus forming immunological complexes. The unbound antibodies were then removed during the washing step. In the second incubation step, the antihuman IgG

conjugated to HRP enzyme was added to the wells and bound to the antibody-antigen complex formed initially. Another washing step is done, removing the unbound conjugate. In the third incubation step, TMB was added. The enzyme mediated cleavage of the substrate resulting in color change. A stop solution was then added to stop the reaction. A change from blue to yellow color indicates a positive reaction while the colourless one represents negative reaction (no antibodies to KSHV/HHV-8).

The colour intensity is proportional to the level of KSHV/HHV-8 IgG antibodies in a sample. The optical densities are then measured spectrophotometrically in a microplate reader (VersaMax; Molecular Devices, Silicon Valley, CA, USA) according to the test instructions. The cut off value turns the measured optical densities into reactive or nonreactive results. The seropositive samples were used for detection of DNA. The negative and positive controls were checked to validate the results. Distribution of KSHV/HHV8-IgG antibodies was stratified according to age, PSA level, and Gleason scores. Significant differences ($p<0.05$) were noted using Chi-square test.

2.4.3 HPV IgG ELISA 4.0 (DRG)

The HPV IgG ELISA (DRG Diagnostics, Germany) was used to screen 187 plasma samples for IgG antibodies following the manufacturer's instructions. It was a semi-quantitative indirect type of ELISA in which the microplates were coated with viral like particles derived from HPV type 6, 11, 16 as well as 18. This ELISA test is composed of two incubation steps. The first incubation step, the solid phase is treated with diluted samples and the anti-HPV IgG are captured by antigens if present. This is followed by a washing step in which all unbound components of the sample are washed out. The second incubation step involves detection of bound anti-HPV IgG by the addition of anti IgG antibody labeled with peroxidase (HRP). An optical density is generated by the enzyme captured on the solid phase, which is acting on the substrate or chromogen mixture.

The colour intensity is proportional to the level of anti-HPV IgG antibodies in a sample. The generated optical signal is proportional to the amount of present in the sample. The optical density was then read according to the test instructions using a microplate reader (VersaMax; Molecular Devices, Silicon Valley, CA, USA). The cut off value turns the measured optical densities into reactive or nonreactive results. The negative and positive controls were checked to validate the results. The seropositive samples were used for detection of DNA. Distribution of HPV IgG antibodies was stratified according to age, PSA level, and Gleason scores. Significant differences ($p<0.05$) were noted using Chi-square test.

2.5 DNA extraction

DNA was extracted from all prostate cancer and BPH plasma samples using Quick-gDNA Miniprep W/Zymo spin IIC (Zymo Research, USA). The manufacturer's instructions were followed. Elution was done in 50µl and then stored at -20^{0}C until use. The extracted DNA was later used for PCR.

2.6 Conventional polymerase chain reaction (PCR)

PCR was performed for the detection of HBV and HHV-8 DNA from plasma DNA. HPV PCR was not done, due to the fact that plasma is not an ideal source of HPV DNA. PCR was done on the extracted DNAs from plasma samples. These PCRs were nested PCR assays which involved two consecutive rounds. The second round primers are specific to a region within the first round primers. All the PCR primers were synthesized from Inqaba Biotech (Pretoria, South Africa).

2.6.1 PCR for HBV

The sense primer (S 5'-GGT TAT CGC TGG ATG TGT-3') and antisense (AS 5'-ACC CAG AGA CAA AAG AAA A-3') were used in the first round reaction and the sense (S3 5'-CTC TTC ATC CTG CTG CTA TGC C-3') and antisense (S4 5'-CAG AC TTG GCC CCC AAT ACC-3') primers were used for nested-PCR to amplify 366 bp of the overlapping polymerase and surface region of HBV (Marrone *et al*., 2005). The final 25µl reaction mixture contained 20µl of mastermix (0.2µM of DNTPs (Invitrogen), 0.2mM of Tris-HCL (pH8.3), 1.07mM of potassium chloride, 0.2ng/µl primers, 0.02U/µl of Taq polymerase (Invitrogen), and 12.28µl of nuclease-free water(Qiagen, Valencia, USA) and 5µl of DNA template per reaction. A positive (a sample containing HBV DNA) and a negative control (nuclease-free water) was used to determine the expected positive band and ensure that there was no contamination. The thermal cycling conditions included 110ºC for heat lid, denaturation at 95ºC for 1 minute, annealing at 55ºC for 1 minute, extension at 72ºC, for 1 minute. This protocol involved 35 cycles for first round PCR and 20 cycles second round PCR. A 1.5% of 10mg/ml agarose stained gel was prepared, and a 100bp molecular marker was used for size confirmation (80v, 300Amp, 35 minutes). The PCR products (5µl) were visualised using a gel documentation system (Syngene G, Germany Version 06-2.d.1).

2.6.2 PCR for HHV-8

The external primers (KSA) 5'-GTCTTTCAGACCTTGTTGG-3'; (KSB) 5'-CCCGTTAGAACAAGTATA-3' were used for first round reaction and internal primers (KS1) 5'-GACCTTGTTGGACATCCTG 3'; (KS2) 5'-GTATTTAGTTTGTGACACGG-3' were used for

nested PCR to amplify a 233 bp segment of the ORF 26 gene (Chang *et al.,* 1994). The final PCR mix was 20µl containing 1.25X PCR buffer, 0.1mM primer, 0.1mM DNTPs (Invitrogen), 1.5mM $MgCl_2$, 0.0025u/µl Taq polymerase (Invitrogen), 2µl DNA template (4µl for nested PCR) and 14µl of nuclease-free water (Qiagen, Valencia, USA). The thermal cycling conditions for the PCR included: 110ºC for the heat lid, initial denaturation at 94ºC for 3 minutes, denaturation at 94ºC for 1 minute, annealing at 57ºC for 1 minute, extension at 72ºC for 1 minute, and final extension at 72ºC for 10 minutes and stored at 4ºC. This protocol involved 35 cycles for both first and second round PCR. A 1.5% of 10mg/ml agarose stained gel was prepared, and a 100bp molecular marker (Thermo Scientific) was used for size confirmation. The PCR products (3µl) were visualised using a gel documentation system (Syngene G, Germany Version 06-2.d.1) with the following settings: 100v, 300Amp, 35 minutes.

2.7. Agarose gel electrophoresis

The gel was prepared by dissolving 1.5g of agarose powder in 100ml of 1x TAE buffer for 3 minutes and dissolved completely in a microwave oven. The melted agarose was then allowed to cool after which 5µl of ethidium bromide (10mg/ µl) was added. The agarose solution was then poured into a cast in which a comb was placed to create wells for loading the samples. Once the gel had solidified, the comb was removed, leaving the wells where DNA samples were to be loaded. One micro litre of 6x orange loading dye was mixed with 5µl of PCR product and loaded. A 100bp ladder (Thermo Scientific) was used for the measurement of the molecular weight of the DNA fragments. The gel was visualised using a gel documentation system (Syngene G, Germany Version 06-2.d.1) with the following settings: 100v, 300Amp, 35 minutes (3µl) PCR product for HHV-8 and 120v, 300Amp, 45 minutes, (3µl) PCR product for HPV.

2.8. DNA purification of PCR products

PCR products were purified using Qiaquick PCR purification Kit (Qiagen, Valencia, USA) following the manufacturer's protocol. The purified PCR products were loaded on a 2% agarose gel for the confirmation of the expected band sizes (running conditions: 100v, 300Amp, 35 minutes, (3µl) PCR product). The purified PCR products were stored at -20^{0}C until use.

2.9. Sequencing and genetic subtyping

Purified amplicons were subjected to Sanger sequencing which is a direct population based sequencing, using nested PCR primers. The sequences were edited manually, assembled

and translated into amino acids using Bioedit software (Hall, 1999). Viral genetic subtypes were obtained using phylogenetic analysis with Molecular Evolutionary Genetics Analysis (MEGA 6.0) (Tamura *et al.*, 2013).

3. Data analysis

The distribution of HBV, HPV and HHV-8 markers was evaluated statistically (by Chi square test) using social science statistics (an online statistical tool). Univarate and multivariate analysis was applied to determine confounding factors. Significant differences were determined by p value calculations. A p value of less than 0.05 ($p<0.05$) was considered significant.

CHAPTER THREE: RESULTS

3.1 Demographic and immunologic data of study participants

A total of 187 prostate cancer (n=76) and benign prostatic hyperplasia (n=79) patients participated in this study, of which 32 did not have BPH or prostate cancer status data. Of these males, 9.0% (n=17) were between the age 40-59 years, 75.9% (n=142) were between the age of 60-79 years, 14.4% (n=27) were between the age of 80-89 years, 0.5% (n=1) was between the age of 100-119 years (specifically 107 years old). The mean age was 71.2 years (range, 49-107 years). The PSA levels were available for only 165 patients while one of the 76 PCA patients did not have the Gleason score respectively. The demographic and immunologic profile of the study participants are summarised in table 6.

Table 6: Demographic data and immunologic profile of study participants.

Age (years)	**No (%)**
40-59	17 (9.1)
60-79	142(75.9)
80-99	27 (14.4)
100-119	1(0.5)
PSA Level	
≤ 4	12 (6.4)
>4	153 (81.8)
No data	22 (11.8)
BPH/PCA	
BPH	79 (42.3)
PCA	76 (40.6)
No data	32 (17.1)
Gleason score	
<6	14 (18.4)
7	18 (23.7)
8 -10	43 (56.6.)
No data	1 (1.3)

PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign Prostatic Hyperplasia, PCA: Prostate cancer

3.2 The seroprevalence of HBV, HPV and HHV-8

The prevalence of HBsAg, HPV IgG antibody, and HHV-8 IgG antibody was 4.8%, 5.3% and 26.6% respectively as shown in table 7. The above mentioned prevalences are indications of exposure to HBV, HPV and HHV-8.

Table 7: Prevalence of hepatitis B virus, human herpes virus type 8 and human papilloma virus serological markers in 187 study participants

VIRUS	Marker	No.	Prevalence (%)
HBV	HBsAg (n=187)	9	4.8
HPV	HPV IgG Ab (n=187)	10	5.3
HHV-8	HHV-8 IgG Ab *(n=123)	33	26.8

HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; HPV: Human papilloma virus; HPV IgG Ab: Human papilloma virus Immunoglobulin G antibody; HHV-8: Human herpes virus type 8; HHV-8 IgG AB: Human herpes virus type 8 Immunoglobulin G antibody *only a total of 123 samples were tested while 64 were not tested due to the insufficiency of the ELISA test.

3.2.1. Prevalence of HBsAg based on age, PSA level, Gleason score and BPH or cancer status by ELISA

There was no statistical significant difference between PCA and HBV infection observed based on age, BPH status, PSA and GS. HBsAg was detected more in individuals with BPH than those with PCA and this was statistically significant at χ^2 =6.0046, P<0.05. The overall prevalence of HBV in the study population was 4.8% (9/187). This is clearly shown in table 8.

Table 8: The distribution of hepatitis B virus seropositivity in 187 study participants (Univarate analysis)

	Total group (n=187)	HBsAg seropositivity	Chisquare	p value
	n= (%)	**n= (%)**	χ^2	**p value**
AGE (years)				
40-59	17(9.1)	2(11.8)	3.2	0.3598
60-79	142(75.9)	7(4.9)		
80-99	27(14.4)	0(0)		NS
100-119	1(0.5)	0(0)		
BPH/PCA				
BPH	79(42.2)	6(7.6)	6.0	0.14269
PCA	76(40.6)	0(0)		
No data	32 (17.1)	3(9.3)		S
PSA				
BPH	68(36.4)	5(7.4)	-	-
PCA	69(37)	0(0)		
No data	50(27)	3(6)		-
GS				
<6	14(18.4)	0(0)	_	_
7	18(23.7)	0(0)		
8-10	43(56.6)	0(0)		
No data	1(1.3)	0(0)		

NS:Not significant, S:Significant PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign Prostatic Hyperplasia, PCA: Prostate Cancer

3.2.2 Prevalence of HPV IgG antibodies based on age, PSA level, Gleason score and BPH or cancer status by ELISA

There was no statistical significant difference between PCA and HPV based on age, BPH status, PSA and GS observed (table 9). The overall prevalence of HPV IgG antibodies was 5.3% (10/187).

Table 9: The distribution of HPV IgG AB seropositivity (Univarate analysis)

	Total Group (n=187)	HPV IgG AB seropositivity		Chi-square
	n= (%)	**n= (%)**	χ^2	**p value**
AGE (years)				
40-59	17(9.0)	3(17.6)	5.6	0.130
60-79	142(75.9)	6(4.2)		
80-99	27(14.4)	1(3.7)		NS
100-119	1(0.5)	0(0)		
BPH/PCA				
BPH	79(42.2)	6(7.5)	0.9	0.33169
PCA	76(40.6)	3(3.9)		
No data	32(17.1)	1		NS
PSA				
BPH	68(55.3)	4(6)	0.2	0.683389
PCA	69(37)	3(4.6)		
No data	50(27)	2(4)		NS
GS				
<6	14(18.4)	0(0)	-	-
7	18(23.7)	0(0)		
8 -10	43(56.6)	3(7)		-
No data	1(1.3)	0(0)		

NS:Not significant, S:Significant; PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign Prostatic Hyperplasia, PCA: Prostate Cancer

3.2.3 Prevalence of HHV-8 IgG antibodies based on age, PSA level, Gleason score and BPH or cancer status by ELISA

There was no statistical significant difference between PCA and HHV-8 based on age, BPH status, PSA and GS observed (table 10). The prevalence of HHV-8 IgG antibodies was 26.6% (33/123), 64 samples were not tested due to insufficient ELISA test.

Table 10: The distribution of HHV-8 IgG AB seropositivity (Univarate analysis)

Total group (n=187)		HHV-8 IgG AB seropositivity	Chisquare	
	n= (%)	**n= (%)**	χ^2	**p value**
AGE (years)				
40-59	10(8.1)	1(10)	4.5	0.212892
60-79	97(78.9)	26(26.8)		
80-99	15(12.2)	5(33.3)		NS
100-119	1(0.8)	1(10)		
BPH/PCA				
BPH	53(43.1)	13(24.5)	0.1	0.823594
PCA	53(43.1)	14(26.4)		
No data	17(13.8)	6(35.2)		NS
PSA				
BPH	45(36.5)	10(22.2)	0.7	0.39261
PCA	43(34.6)	13(28.9)		
No data	35(15.5)	10(28.45)		NS
GS				
<6	6(11.3)	1(16.7)	1.6	0.4522634
7	8(15)	1(12.5)		
8-10	38(7.7)	12(31.6)		NS
No data	1(1.3)	0(0)		

NS: Not significant, S: Significant; PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign Prostatic Hyperplasia, PCA: Prostate Cancer

3.3. Prevalence of HBV infection by PCR

There was no statistical significant difference between PCA and HBV based on age, BPH status, PSA and GS observed. Polymerase chain reaction was applied to detect HBV DNA HBsAg positive samples. The expected fragment of 366bp was obtained in 3/9 (33.3%) samples tested. Figure 14 shows a gel image of an expected fragment of 366bp.

3.3.1 Prevalence of HBV occult infection

There was no statistical significant difference between PCA and HBV occult infection based on age, BPH status, PSA and GS observed. Polymerase chain reaction was applied to detect occult HBV infection in HBsAg negative samples. Occult HBV was assessed based on the presence of detectable HBV-DNA in HBsAg negative samples. A high prevalence of occult infection was observed; 12.9% (23/178). A gel image showing an expected fragment of 366bp is shown in figure 15.

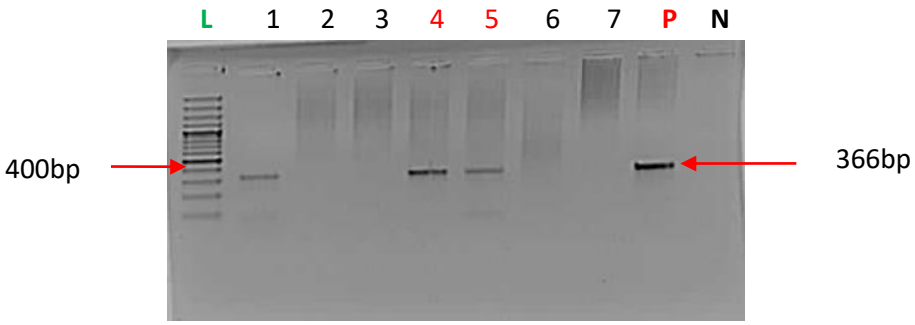

Figure 14: Amplification of HBV DNA. Lanes **L**, **P** and **N** represent the molecular weight marker (100bp), positive control and negative control respectively. Lane 1, 4, 5, represent amplification of HBV occult infection. Lanes 2, 3, 6 and 7 represent the absence of HBV DNA meaning that there is no occult infection for those specific patients respectively. The negative control showed no band which means that there was no contamination.

3.3.2 Prevalence of HBV occult infection based on age, PSA level, Gleason score and BPH or cancer status

There was no statistical significant difference between PCA and HBV occult infection based on age, BPH status, PSA and GS observed (table 11). Twenty three of the 178 HBsAg negative samples (23/178, 12.9%) had HBV occult infection.

Table 11: The distribution of HBV occult infection based on age, PSA, Gleason score, BPH or cancer status

	Total Group (n=187)	HBV occult Infection	Chisquare	P value
	n= (%)	n= (%)	χ^2	p value
AGE (years)				
40-59	15(8.4)	4(26.7)	4.2	0.240696
60-79	135(75.9)	14(10.4)		
80-99	27(15.2)	5(18.5)		NS
100-119	1(0.6)	0(0)		
Total	178			
BPH/PCA				
BPH	73(41)	10(13.7)	0.1	0.734131
PCA	76(42.7)	9(11.8)		
No data	29(16.3)	4(1.4)		NS
PSA				
BPH	12(6.7)	0(0)	1.6	0.207366
PCA	144(80.9)	17(11.8)		NS
No data	22(12.4)	6(27.3)		
GS				
<6	14(18.4)	1(7.1)	0.2	0.892319
7	18(23.7)	2(11.1)		NS
8 -10	43(56.6)	5(11.62)		
No data	1(1.3)	0(0)		

NS:Not significant, S:Significant; PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign prostatic Hyperplasia, PCA: Prostate cancer

3.3.3 Prevalence of HBV DNA based on age, PSA level, Gleason score and BPH or cancer status

Polymerase chain reaction was applied to detect HBV DNA in HBsAg positive samples. The prevalence of HBV was 33% (3/9). The expected fragment of 366bp was obtained in 3/9 samples tested. A representative gel image is shown in figure 15.

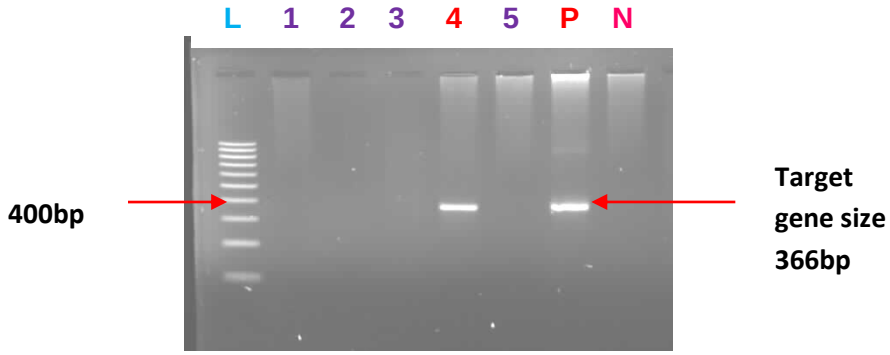

Figure 15: Amplification of HBV DNA. Lanes **L**, **P** and **N** represent the molecular weight marker (100bp), positive control and negative control respectively. Lane **4** represents amplification of HBV DNA (366bp). Lanes **1**, **2**, **3**, **5** represent the absence of HBV DNA for those specific patients respectively. The negative control showed no band which means that there was no contamination.

3.4 Prevalence of HHV-8 DNA amplification based on age, PSA level, Gleason score and BPH or cancer status

No association was observed between PCA and HHV-8 infection based on age, BPH status, PSA and GS was observed (table 12). Polymerase chain reaction was applied to detect HHV-8 DNA from both HHV-8 Ab negative and positive samples. The prevalence of HHV-8 DNA was 8.5% (16/187). The expected fragment of 233bp was obtained in 16/187 samples tested. A representative gel image is shown in figure 16.

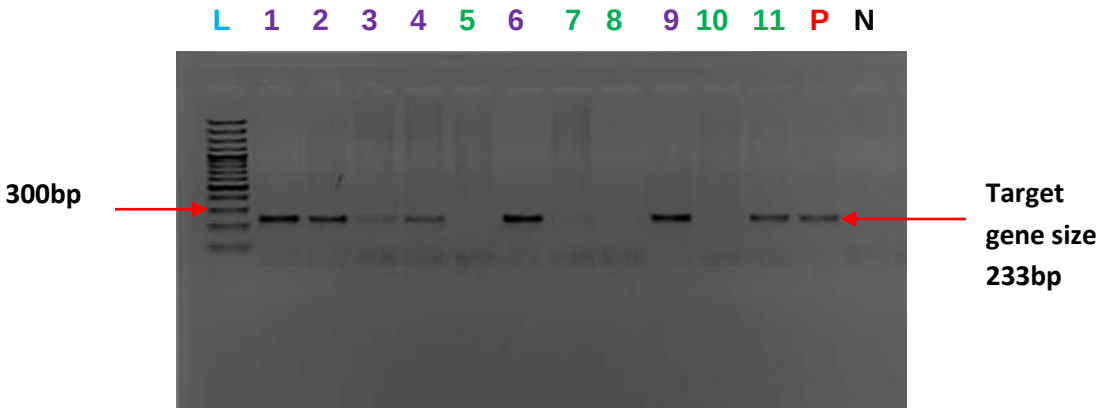

Figure 16: Amplification of HHV-8 DNA (ORF 26). Lanes **L**, **P** and **N** represent the molecular weight marker (100bp), positive control and negative control respectively. Lanes **1**, **2, 3, 4, 6, 9** and **11** represents amplification of HHV-8 ORF 26 DNA (233bp). Lane **5, 7,8,10** represent the absence of HHV-8 DNA for those specific patients respectively. The negative control showed no band which means that there was no contamination.

Table 12: The distribution of HHV-8 infection based on age, PSA, Gleason score, BPH or cancer status (Univarate analysis)

	Total group (n=187)	HHV-8 PCR	Chisquare	p value
	n= (%)	**n= (%)**	**χ^2**	**p value**
AGE (years)				
40-59	17(9.0)	2(11.8)	61.1	0.0001
60-79	142(75.9)	11(7.8)		
80-99	27(14.4)	3(11.1)		S
100-119	1(0.5)	0(0)		
BPH/PCA				
BPH	79(42.2)	6(7.6)	1.3	0.25514
PCA	76(40.6)	10(13.2)		NS
No data	32 (17.1)	0(0)		
PSA				
BPH	68(36.4)	6(8.82)	1.07	0.301593
PCA	69(37)	10(15)		NS
No data	50(11.8)	2(4)		
GS				
<6	14(18.4)	2(14.3)	0.9	0.627329
7	18(23.7)	1(5.6)		NS
8 -10	43(56.6)	6(14)		
No data	1(1.3)	1(6.4)		

NS: Not Significant, S: Significant; PSA: Prostate Specific Antigen, GS: Gleason Score, BPH: Benign prostatic Hyperplasia, PCA: Prostate Cancer

3.5 Purification of PCR amplicons

Sixteen of the 19 (84.2%) successfully amplified HHV-8 (ORF 26 gene) samples were subjected to purification. A representative gel image is shown in figure 17.

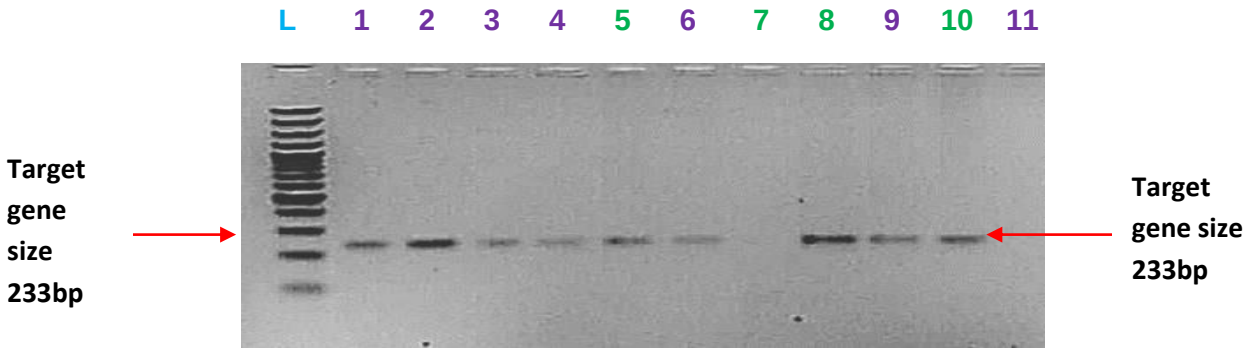

Figure 17: Purification of HHV-8 DNA (ORF 26) amplicons. Lane **L**, represent the molecular weight marker (100bp). Lanes **1** to **11** represents amplification of HHV 8 ORF 26 DNA (233bp). One microliter of the purified PCR amplicons was loaded on a 2% agarose gel electrophoresis. The purification process worked well, as strong bands can be observed when only 1µl of the purified amplicons were loaded into a 2% agarose gel.

3.6. Associations between HPV, HBV and HHV-8 coinfection based on serology and amplification

The association between PCA and HHV-8, HPV, HBV infection (by serology and amplification) based on PCA stage was evaluated. The observation based on HBV infection was statistically insignificant as shown in table 13. There was no sample which was reactive for all the three serological markers together. The presence of viruses in PCA patients may contribute to the disease.

Table 13: Associations between HPV, HBV and HHV-8 coinfection based on serology and amplification.

Virus	Total group (n=187)		Chisquare	P value
HHV-8	**n= (%)**	**n= (%)**	χ^2	**P value**
<6	14(18.4)	1(7.1)	0.9	0.62733
7	18(23.7)	2(11.1)		NS
8-10	43(56.6)	5(11.62)		
No data	1(1.3)	0(0)		
HBV				
<6	14(18.4)	0(0)	-	-
7	18(23.7)	0(0)		
8 -10	43(56.6)	0(0)		
No data	1(1.3)	0(0)		
HBV + HHV-8 (1)				
***N0082**				
HPV + HHV-8 (1)				
***N0082**				
HBV + HPV (1)				
***N00119**				
PCR RESULTS				
HBV				
<6	14(18.4)	1(7.1)	0.2	0.89232
7	18(23.7)	2(11.1)		NS
8 -10	43(56.6)	5(11.62)		
No data	1(1.3)	0(0)		
HHV-8				
<6	14(18.4)	2(14.3)	0.9	0.62733
7	18(23.7)	1(5.6)		NS
8 -10	43(56.6)	6(14)		
No data	1(1.3)	1(6.4)		
HBV + HHV-8 (2)				
***N0051 and N0082 are BPH samples, thus no Gleason scores.**				
The fact that the samples N0051, N0082 and N0119 are BPH samples does not allow us to assess the association of these viral infections (HBV, HHV-8, and HPV) with PCA stage.				

*BPH: Benign prostatic Hyperplasia, PCA: Prostate cancer

3.7 Summary of seroprevalence and DNA prevalence

A high HBV occult infection prevalence was observed in the current study (table 14). More ELISA overlaps with PCR where observed in HHV-8 than HBV.

Table 14: Summary of seroprevalence and amplification in the study population

Virus	**Total tested**	**Antibody Prevalence (ELISA)**	**PCR prevalence (Viral DNA)**	**ELISA and PCR overlaps**
HBV	187	9/187=4.8%	3/9 =33.3%	3
			occult infection *****23/178=**12,9%**	
HHV-8	123	33/123= 26.6%	9.4%	5

***** A high occult infection prevalence was observed.

3.8 Phylogenetic analysis

The samples which were successfully purified after PCR for the HBV overlapping surface polymerase gene as well as HHV-8 ORF26 gene were Sanger sequenced. These sequences were then subjected to Basic Local Alignment Search Tool so as to confirm if the HBV and HHV-8 DNA sequences aligned with other HBV and HHV-8 reference sequences and to check for contamination. These sequences were then aligned against reference sequence of the viral full genome. A consensus was generated from these sequences as shown in figure 18 and 20. Figure 19 and 21 shows the predicted amino acid sequences for HBV and HHV-8 respectively.

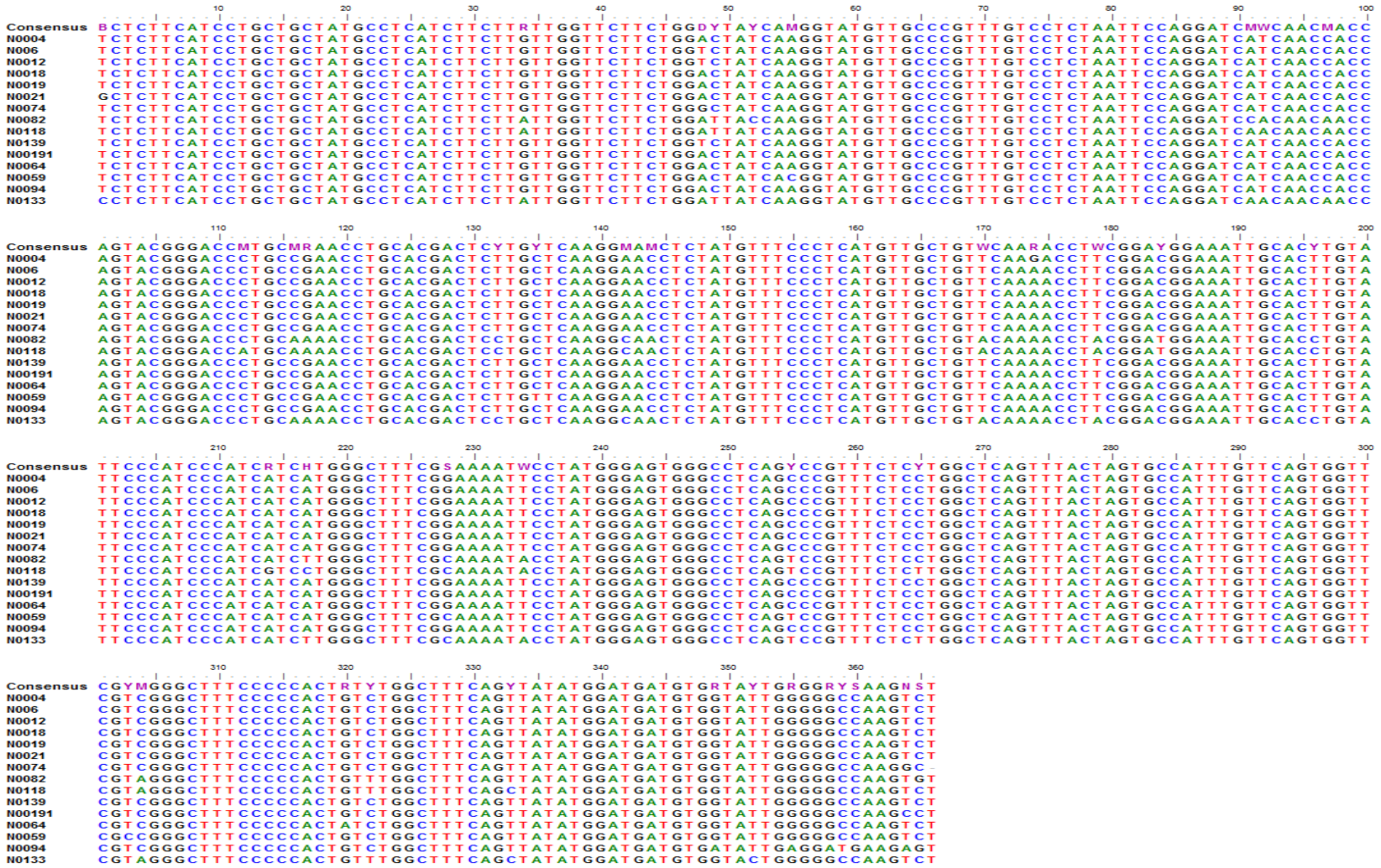

Figure 18: Predicted nucleotide sequences of the 366bp HBV overlapping surface polymerase region of 15 prostate cancer and benign prostatic hyperplasia South African patients. The sequences were aligned against a consensus generated from the sequences. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 50% on BIOEDIT (V.7.2.5).

Figure 19: Predicted amino acid sequences of HBV in prostate cancer and benign prostatic hyperplasia patients of South Africa. The sequences were aligned against a consensus generated from the sequences. The dots indicate identical amino acids and the changed amino acids are shown. The dots indicate identical amino acids and the changed amino acids are shown. The amino acid isolates differs from the consensus at positions such as S1A, L11V. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 50% on BIOEDIT (V.7.2.5).

Figure 20: Predicted amino acid sequences of the 233bp HHV-8 ORF26 gene of 11 prostate cancer and benign prostatic hyperplasia South African patients. The sequences were aligned against a consensus generated from the sequences. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 95% on BIOEDIT (V.7.2.5).

Figure 21: Predicted amino acid sequences of HHV-8 in 11 prostate cancer and benign prostatic hyperplasia patients of South Africa. The sequences were aligned against a consensus generated from the sequences. The dots indicate identical amino acids and the changed amino acids are shown. The amino acid isolates differs from the consensus at positions V21A and H38R. The sequences were aligned using Clustal W multiple alignment with threshold frequency of 50% on BIOEDIT (V.7.2.5).

3.9 HBV and HHV-8 genotypes

There was no association observed between PCA and HBV genotypes as well as HHV-8 genotypes. Among the 23 patients with detectable HBV DNA, 15 samples were successfully sequenced. These sequences were genotyped using HBV database [HBVdb], and HBVseq program from HIV Stanford database. Two genotypes were found in this study population. HBV genotype E (n=13, 86.7%) and A (n=2, 13.3%). A phylogenetic tree (Figure 22) was constructed using MEGA6 which confirmed the genotyping results obtained from the above mentioned databases.

All the 11 samples with detectable HHV-8 DNA, were successfully sequenced. These sequences were confirmed to be of HHV-8 using BLAST. Two genotypes were found in this study population, genotype K (n=10, 90.9%) and R (n=1, 9.09%). A phylogenetic tree (Figure 23) was constructed using MEGA6 which confirmed the genotyping results.

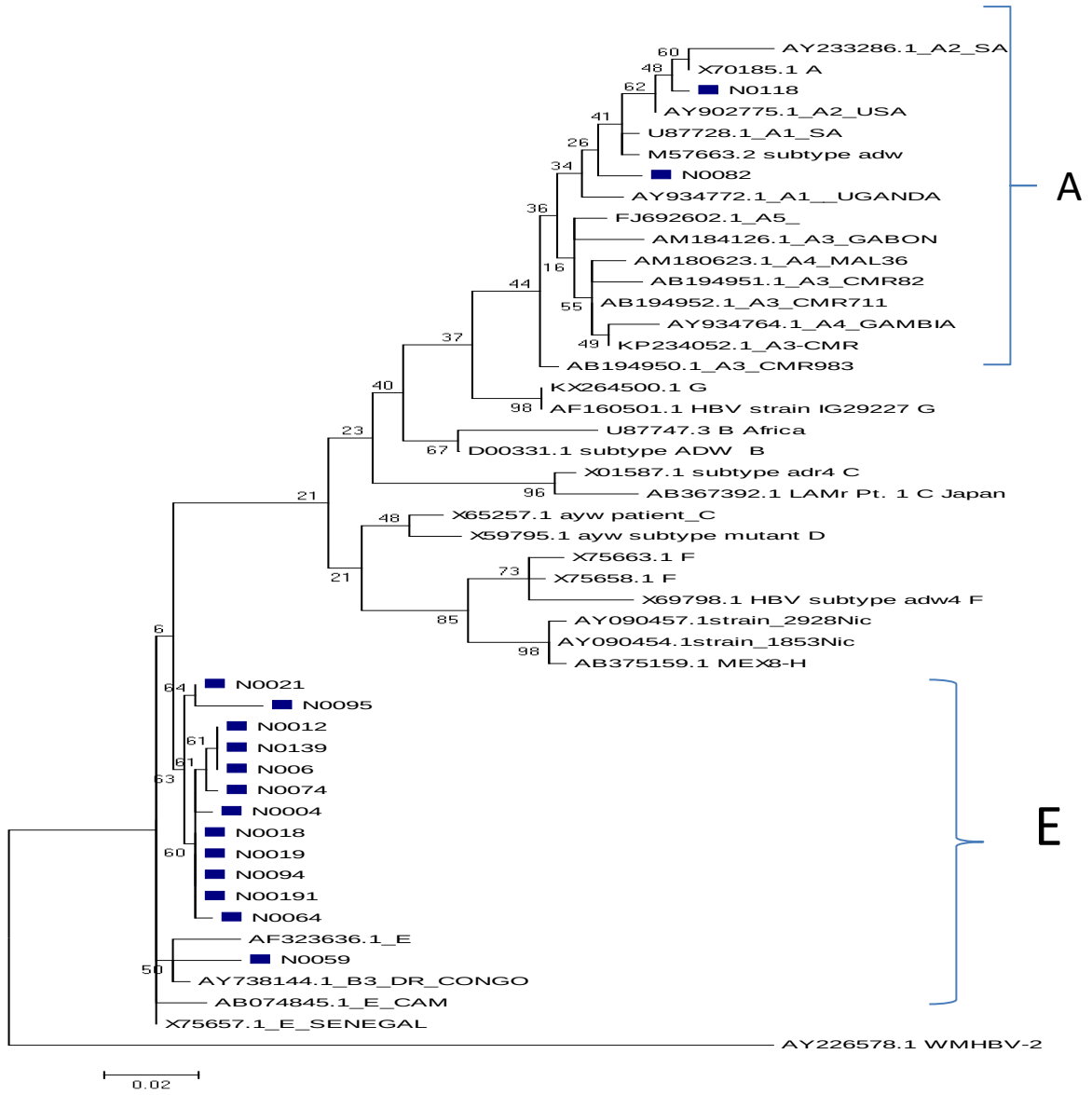

Figure 22: Phylogenetic relationships of the 366bp overlapping surface polymerase gene of HBV analysed using MEGA6 software. A woolly monkey HBV sequence (AY226578) from GenBank was used as out-groups. The tree was constructed using the Maximum likelihood method. The test samples are indicated with a blue square. GenBank accession numbers and the country of origin of reference sequences are also indicated.

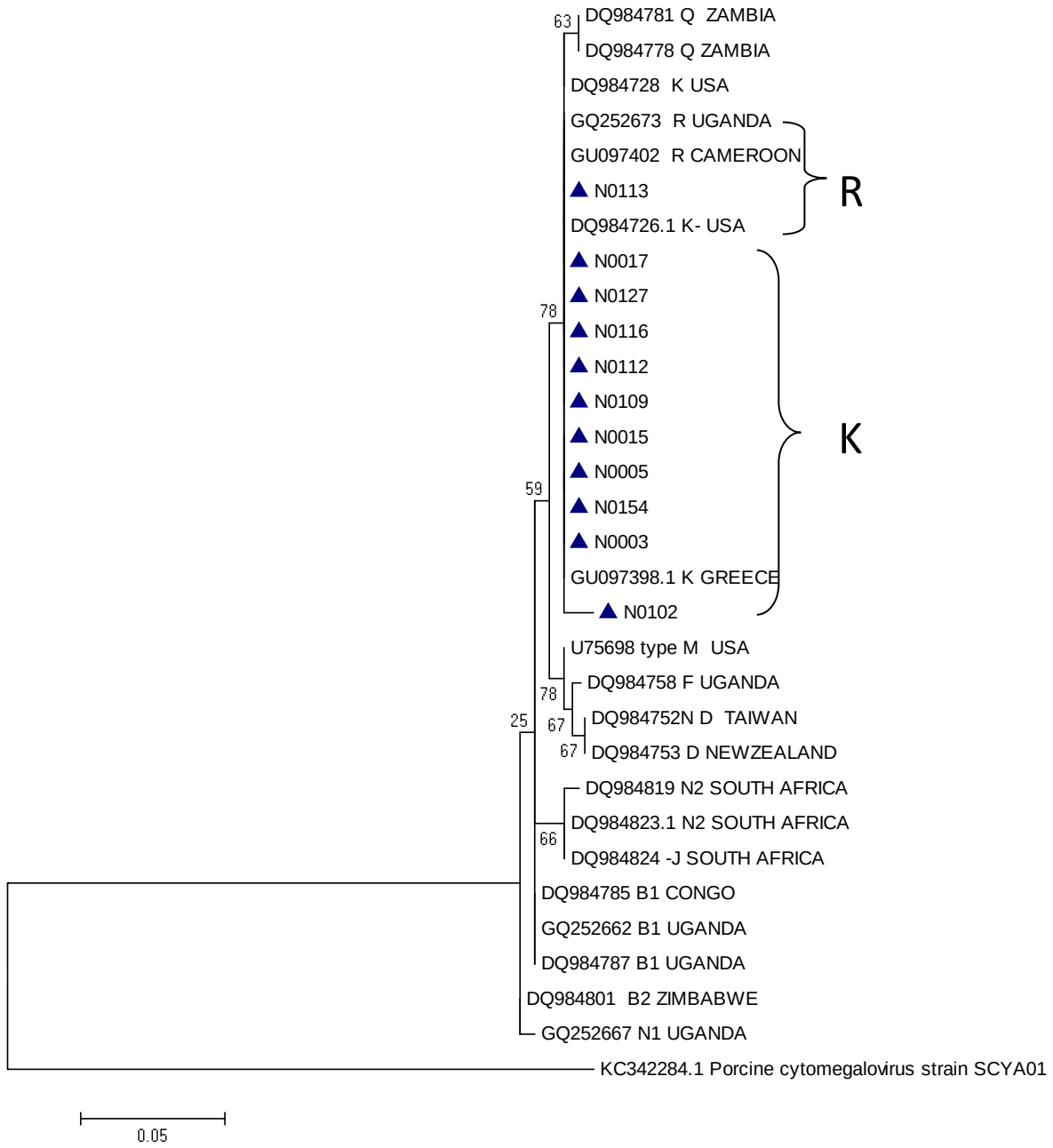

Figure 23: Phylogenetic relationships of the 233bp ORF26 gene of HBV analysed using MEGA6 software. A porcine CMV strain sequence (KC342284.1) from GenBank was used as out-group. The tree was constructed using the Maximum likelihood method. The test samples are indicated with a blue triangle. GenBank accession numbers and the country of origin of reference sequences are also indicated.

CHAPTER FOUR: DISCUSSION AND CONCLUSION

Several reports have suggested the association of viruses in the pathogenesis of prostate cancer (Carrillo-infante *et al*., 2007; Ge *et al*., 2013; Jha *et al*., 2016). The current study was aimed at identifying HBV, HHV-8 and HPV, implicated in other forms of cancer, in a cohort of South African patients with either prostate cancer (PCA) or benign prostatic hyperplasia (BPH); and to seek possible associations thereof.

4.1 HBV prevalence in the study cohort

The HBV prevalence was determined by screening for HBsAg in plasma of PCA and BPH patients. The observed HBsAg prevalence among the studied population was 4.8% (9/187) which is an intermediate prevalence level (2–7%) according to WHO classification (WHO, 2015).

A seroprevalence of 4.8% was observed in another South African study by Firnhaber *et al*., (2008), although this was in an HIV positive population. Data on HBV seroprevalence in prostate cancer patients in South Africa is not available or limited. However, different levels of infection have been reported for other target groups in South Africa. For example, Firnhaber *et al,* (2008) noted a similar prevalence of 4.8% in an HIV infected cohort, while Duacancelle *et al*, (2013) reported up to 44% among an HIV population. Elsewhere, a 4.2% prevalence was noted among oncology patients in Turkey (Kose, 2011), and 9.7% among pregnant women in Cameroon (Frambo *et al*., 2014). In general, it appears the prevalence of HBV reflects the risk factors of the geographic location or the specific population under study. Univarate analysis did not reveal any statistical significant difference in the distribution of HBV when stratified according to Gleason score, prostate specific antigen and age. However, HBsAg was detected more in individuals with BPH than those without and this was statistically significant at (χ^2=6.0, p< 0.05). The meaning of this observation is not clear from the current study.

HBV occult infection (OBI) defined as the persistence of HBV DNA in the liver tissue of HBsAg negative individuals, (Conjeevaram *et al*., 2001); is variably prevalent in different parts of the world which are endemic for HBV infection (Mphahlele *et al*., 2006, Gutierrez-Garcia *et al*., 2011, Powel *et al*., 2014). OBI was assessed based on the presence of detectable HBV DNA in HBsAg negative samples. In the present study, a 12.9% prevalence of OBI was observed. OBI results from a continuous suppression of viral replication and gene expression (Baghabanian *et al*., 2016). More than 20% of OBI patients present no serological markers because the antibody titer may become undetectable over time, leaving HBV DNA as the only marker of infection (Zobeiri, 2013).

A search of literature did not produce previous data on OBI in prostate cancer and/or BPH patients. Nevertheless, a 33.7% OBI has been observed in an HIV population in Limpopo (Ayuk *et al*., 2013), and 20.4% in West Africa (Magoro *et al*, 2016). Univarate analysis did not show any statistical significant difference in the distribution of HBsAg when stratified according to Gleason score, prostate specific antigen, age and BPH status. When OBI and HBsAg are combined, the overall prevalence was 17.7%. According to the WHO classification, an 8% HBsAg prevalence is considered high. Therefore, the observed level of active HBV infection is high in the current study population. This finding sheds light on the high level of undetected or missed HBsAg by routine tests such as serology; this can assist in terms of treatments given to individuals who have prostate cancer with OBI. It's therefore significant to check for OBI in PCA patients.

The sequences derived from amplified HBV DNA were analyzed for genotypes. This study reports a high prevalence of HBV genotype E infection (86.7%), followed by HBV genotype A (13.3%) in prostate cancer and BPH patients of South Africa. HBV genotype E is most prevalent in West Africa, while genotype A is most prevalent in Southern Africa (Kew, 2008; Mora *et al*., 2010). According to our knowledge, there has not been any report on HBV genotype E in OBI patients of South Africa. Other studies have reported up to 99.9% (Cameroon), 85.7% (Angola) prevalence of genotype E (Magoro *et al*., 2016; Ducanecelle *et al*., 2013; Valente *et al*., 2010). It is worthwhile to note that such a high prevalence of HBV genotype E was observed in South Africa, whereas it is known to be common in West Africa. The reason for this observation could not be inferred from the current study.

The prevalence of HBV genotype A (13.3%) was observed in the present study. HBV genotype A predominates in Southern Africa and most parts of sub-Saharan Africa (Kimbi *et al,* 2004; Kew, 2008; Mora *et al*., 2010). Genotype A has a four-fold greater hepatocarcinogenic potential than the non A genotypes of HBV. It accounts for 75% of HBV isolates in Southern Africa (Kew *et al*., 2005)

4.2 HHV-8 prevalence in the study cohort

The HHV-8 prevalence was determined by screening for HHV-8 IgG antibodies in plasma of prostate cancer and BPH patients. A 27% seroprevalence was observed in the current study population.

A seroprevalence range of 32-100% has been observed for the general population of South Africa (Klaskala *et al*., 2005, Isaacs *et al*., 2016). However, in the HIV positive population a contrasting 13.7% (South Africa) was observed. Elsewhere, a 20.4% (Cameroon), 45.7% (Iran), 39.9% (Tobago) and 15.4% (China) prevalence have been observed in the HIV

positive population (Hoffman *et al*., 2003; Dollard *et al*., 2010; Jalilvand *et al*., 2011; Zhang *et al*., 2011). Univarate analysis did not show any statistical significant difference in the distribution of HHV-8 IgG antibody when stratified according to Gleason score, prostate specific antigen, age and BPH status. This observation is similar to that shown by Sutcliffe *et al*., (2015) which does not support an association between HHV-8 seropositivity and PCA. This is in contrast with many of the previous studies done in this regard such as one done by Hoffman *et al*., (2003) which supports an association between HHV-8 and PCA.

The prevalence of HHV-8 was assessed based on the presence of detectable HHV-8 DNA in HHV-8 IgG antibody positive and negative plasma, 8.5% prevalence of HHV-8 DNA was observed. Antibodies show exposure to infection and the infection may clear out over time; thus a low HHV-8 DNA prevalence was observed as compared to the seroprevalence 27%. Univarate analysis did not reveal any statistical significant difference in the distribution of HHV-8 DNA when stratified according to Gleason score, prostate specific antigen and BPH. However, HHV-8 DNA was detected more in individuals in the 60-79 years age range and this was statistically significant at χ^2=61.1, p< 0.05.

The successfully amplified HHV-8 DNA were analyzed for genotypes, and genotypes K and R were conferred. Although there is limited data on the prevalence of HHV-8 genotypes in South Africa (Isaacs *et al.,* 2016), genotypes A5 and B are considered to be more prevalent. Apparently, this is the first report of genotypes K and R from South Africa. It could be of interest to elucidate the complete genome of the detected genotypes K and R for vaccine design imperatives.

4.3 HPV prevalence in the study cohort

The HPV prevalence was determined by screening for HPV IgG antibodies in plasma of prostate cancer and BPH patients. The observed prevalence among the studied population was 5.3%.

The observed seroprevalence in this study is much less than that found in the general population (64%) of South Africa (Katz *et al*., 2013). Elsewhere, (Iran), a 10% prevalence amongst prostate adenocarcinoma patients was observed (Mokhtari *et al*., 2013); however amongst other study groups such as HIV positive patients a 13.4% and 8.9% (China), where observed (Korodi *et al*., 2005; Yang *et al*., 2015). Univarate analysis did not show any statistical significant difference in the distribution of HPV IgG antibody when stratified according to Gleason score, prostate specific antigen, age and BPH status. This is in contrast with a study done by Mokhtari *et al*., (2013), in Iranian men with prostatic

adenocarcinoma, showed a significant association between prostate cancer and HPV infection; this suggested that HPV may contribute to the increased risk of prostate cancer.

4.4 Limitations of the study

This study had several limitations. Firstly, other serological markers of HBV; such as antiHBs, E-antigen, anti-HBe, anti-HBc were not determined. The missing data would have allowed the classification of patients as either exposed, protected or the degree of active HBV infection. Secondly, it would have been of value to determine the presence of HBV, HPV, and HHV-8 in prostate cancer biopsies. As a result it is not possible with the current data to discuss association of these viruses with cancer growth. This is an important aspect for future investigations.

4.5 Conclusion

The current study has demonstrated for the first time, the presence of genotypes K and R of HHV-8 in South Africa. This study also suggests that there is a high level of occult genotype E HBV infection. Future studies will explore the virome in prostate cancer biopsies.

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