IMPACT OF HOST GENETIC POLYMORPHISM AT IL-6 GENE PROMOTER AT POSITION -174G/C ON THE OCCURENCE OF HBV AND HCV AMONG HIV/AIDS PATIENTS IN LIMPOPO PROVINCE, SOUTH AFRICA

A Dissertation Submitted in Fulfillment of the Requirements for Master of Science Degree in Microbiology

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DECLARATION

I Gogela Ndiitwani Noel, student no: 11606665 hereby declare that this dissertation submitted for the Degree MSc in Microbiology at University of Venda, is my original work and has not been submitted to this or any other institution of higher education. I further declare that all sources cited or quoted are indicated and acknowledged by means of a compiled list of references.

Student: Gogela Ndiitwani Noel

Signature: Gogela N.N Date: 06-06-2017

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Despite the fact that it's just my name that shows up on the front of this dissertation, a large number of individuals have added to its creation. I owe my appreciation to each one of the individuals who made it conceivable by contributing decidedly to make this dissertation a win.

Most importantly I would want to express gratitude toward God for He has presented to me this far.

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DEDICATION

This dissertation is dedicated to my parents Grace and Richard Gogela.

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RESEARCH OUTPUT

The following manuscripts have been prepared for submission to accredited journals

Gogela N.N., Ramalivhana J.N., and Samie A. Sero-prevalence of HCV and HBV in relation to IL-6 serum levels among HIV/AIDS patients in Limpopo province, South Africa. To be submitted to BMC infectious diseases.

Gogela N.N., Ramalivhana J.N., and Samie A. Impact of Host genetic polymorphism at IL-6 promoter gene (-174G/C) on the occurrence of HCV and HBV among HIV/AIDS participants in Limpopo province, South Africa. To be submitted to Genetics and Molecular Research.

Related Conference Presentations

Gogela N. N. and Samie A. Seroprevalence of *E. histolytica* in relation to host genetic polymorphisms at interleukin-6 gene. **2nd WSU-UNIVEN international conference**. October 2016, Polokwane. Oral presentation.

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List of abbreviations

AIDS	: Acquired immune deficiency syndrome
CD4+	: Cluster of differentiation 4
CD8	: Cluster of differentiation 8
CDC	: Centers for Diseases Control and Prevention
CSF	: Cerebrospinal fluid
DNA	: Deoxyribonucleic acid
dNTP	: Deoxynucleotide triphosphate
ELISA	: Enzyme linked immunosorbent assay
HBsAg	: Hepatitis B surface Antigen
HBV	: Hepatitis B virus
HCC	: Hepatocellular carcinoma
HCV	: Hepatitis C virus
HCVAb	: Hepatitis C virus Antibody
HIV	: Human immunodeficiency virus
IL13	: Interleukin 13
IL4	: Interleukin 4
IL5	: Interleukin 5
IL-6	: Interleukin-6
LNFPIII	: Lacto-N-fucopentaose III
MHC	: Major histocompatibility complex
NATSTAL	: National Survey of Sexual Attitudes and Lifestyles

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NF-κB	: Nuclear factor kappa beta
NK	: Natural killer cells
OD value	: Optical density value
PAMPS	: Pathogen associated molecular patterns
PBS	: Phosphate Buffered Saline
PCR	: Polymerase chain reaction
PID	: Pelvic inflammatory disease
PRR	: Pattern recognition receptors
RE	: Restriction enzymes
SNP's	: Single nucleotide polymorphisms
SPSS	: Statistical Package for social science
STAT 6	: Signal transducer and activator of transcription 6
STIs	: Sexually transmitted infections
TCR	: T cell receptor
TH1	: T helper 1 cells
TH2	: T helper 2 cells
TLR	: Toll like receptors
WHO	: World Health Organization

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Abstract

Background: Chronic Hepatitis B (HBV) and hepatitis C (HCV) viruses are among the most commonly known viruses worldwide. The prevalence rates vary greatly from one region to another and over time, hence surveillance studies are needed to monitor the prevalence patterns of these viruses and to implement appropriate preventive measures. Fortunately, only subsets among acutely infected individuals develop a persistent disease suggesting that genetic susceptibility may influence the establishment of chronicity. Cytokines play an important role in the regulation of the immune response. In hepatitis viral infections, cytokine levels may influence the outcome of infection. Polymorphisms in cytokine genes have also been associated with different expression levels in response to infections. Therefore, the present study aims at investigating the potential impacts of host genetic polymorphisms at IL-6 gene promoter (-174G/C) on the occurrence of HCV and HBV among HIV patients in the Limpopo Province of South Africa.

Methodology: Patients were recruited from different hospitals and clinics from three Districts in Limpopo Province. About 466 blood samples were collected and screened using ELISA for anti-HCVAb and HBsAg. DNA was extracted from buffy coat using the Gen-Elute ^{TM} blood genomic kit from Sigma Aldrich. Genotyping of IL-6 -174G/C was carried out using RFLP-PCR. IL-6 genotyping results were confirmed by sequencing. IL-6 serum levels were measured using commercially available ELISA for human IL-6 from MABTECH. SPSS was used to analyze the results. The results were considered to be statistically significant when the p-value obtained is less than 0.05.

Results: Most of the study participants were females (56.4%). Majority of our study participants were aged between 21 to 45 years (52.4%). About 30% of our study participants had a CD4 cell

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count of 251-500. Most of the study participants were from Capricorn district (38.2%). The overall prevalence of HCV and HBV were 15.4% and 20.9% respectively. Highest prevalence of both HCV and HBV was observed in males. The age group >45years had high prevalence of both HCV and HBV although the results was not significant. The infections were not associated with any of the socio-demographic characteristics such as age, gender CD4 cell count an origin. The overall genotypic distribution of IL-6 gene was 35.5% for CC, 24.2% for GC and 18.0% for GG. The genotypes obtained did not show any association with the demographic characteristics within the studied population as well as the reported infections. Participants who were positive for HCV had low levels of IL-6 (81.4%) as compared to those who were HCV negative (72.0%), Participants who were positive for HBV had high significantly levels of IL-6 as compared to those who were HBV negative, the results were statistically significant (**p=0.001**).

Conclusions: The present study demonstrated a high prevalence of both HCV and HBV in the Limpopo Province. The prevalence was higher in males than in females for both HCV and HBV. Both infections were high in participants with age group >45 years. IL-6 -174G/C polymorphism did not show any association with either infection, suggesting that the polymorphism might not have an impact on the occurrence of the reported infections. The higher prevalence was observed among males, which calls for an urgent need for males to be screened for STI's and campaigns for males to use condoms if they have multiple partners.

Key words: Interleukin-6, HCV and HBV

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CHAPTER ONE:

INTRODUCTION AND OBJECTIVES

1.1. Introduction

Chronic hepatitis B and C are among most debilitating medical issues around the world, with high prevalence in subtropical and intertropical districts of the world (Hwang and Choong, 2011 and Lavanchy, 2011). It has been reported that only 5 % of adults infected with hepatitis B present with liver disease while out of the patients infected with hepatitis C, 70% persist and present with the disease (Villa et al., 2011). Over the long haul, both infections will frequently bring about liver intricacies, for example, fibrosis, cirrhosis and, at last hepatocellular carcinoma (Villa et al., 2011). It has been recommended that host hereditary components influence the result of imperceptible reaction to hepatitis viral infection and influence the disease progression (Van Weel *et al.*, 2012). Also, it is outstanding that, contingent upon ethno-topographical foundation of contemplated populaces, there is a striking heterogeneity in incessant hepatitis result and in addition in light of treatment (Chevaliez and Asselah, 2011). In any case, in spite of completely extraordinary atomic physiology and replication procedures, HBV and HCV still portrays the same pathologies profile and lead to the same disease complications, suggesting that disease progression is primarily controlled by immune response and host-virus interactions (Gottwein and Cullen, 2008 and Randall et al., 2007).

HCV and HBV infections commonly induce immune reactive inflammation (Fabricio-Silva et al., 2013). Several risk factors have been investigated with the aim of identifying patients who may be predisposed to a less favorable outcome. Hereditary reviews as a rule, and especially genome wide

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affiliation studies performed in the last decade have demonstrated that hereditary polymorphisms, generally single nucleotide polymorphisms (SNPs) balance host's immune response to infection (Rauch et al., 2010 and Romero-Gomez et al., 2011)

Cytokines affect the natural course of HCV and HBV infection (Amini and Poustchi, 2012). Cytokines are key immune modulatory molecules which deals with the activities of various target cells by method for definitive to specific receptors and are incorporated into the pathogenesis of various (Amini and Poustchi, 2012). Interleukin-6 (IL-6) is a multifunctional cytokine produced by lymphoid and non-lymphoid cells and has been portrayed in the literature as a standout amongst the most imperative controllers of the cytokine-related artful infection (Lukaszewicz et al., 2007). IL-6 is a powerful pleiotropic incendiary cytokine that is viewed as a key development and against apoptotic variables (Lin et al., 2007). The IL-6 is of particular interest because it is expressed in the epithelial cells (Pine et al., 2011).

Hereditary polymorphisms are accounted for to be in charge of between individual variety and assorted qualities and in this manner have been as of late considered as the principle hereditary components required in the advancement and movement of normal and complex illnesses (Van Well *et al*., 2012). It has been reported that IL-6 stimulates the inflammatory and auto-immune processes in many diseases such as diabetes, systemic lupus, prostate cancer, Behçet's disease (Smith et al., 2001 and Tackey et al., 2004). A few reviews have exhibited that - 308TNFa, - 174IL6, and - 1082IL10 might be related with the chronicity of hepatitis B, hepatitis C and the danger of dynamic advancement of fibrosis (Fishman et al., 1998; Knapp et al., 2003 and Cussigh et al., 2011). The present aims to determine the impact of host genetic polymorphism at IL-6 on th.e occurrence of HCV and HBV HIV/AIDS patients in Limpopo province.

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1.2. Study rationale

The common result in HBV and HCV disease changes significantly among people. Disease with HCV and HBV is self-restricted in a blessed minority, while for the larger part of subjects they create chronic infection (Alter et al., 2003 and Seeff 2002). In spite of the fact that contamination with HBV in grown-ups advances to the unending stage in around 5% (Bwogi et al., 2009), among those people with determined HBV or HCV disease, the larger part create constant hepatitis (CH), dynamic fibrosis and even liver tumor (Hoffmann et al., 2001 and Asselah et al., 2007). Be that as it may, there are a few cases that never advance into any noteworthy liver ailment inside the patient's common life expectancy (Poynard et al., 1997 and Kenny-Wash et al., 1999).

It stays obscure why patients tainted with HBV and additionally HCV much of the time swing to be incessant and the result of HBV and additionally HCV disease drastically fluctuates. Other than the pathogenesis of viral elements, the most imperative component is the distinctive safe reaction to HBV or HCV contamination between people. The individual vulnerability and the result of HCV and HBV diseases have been related to polymorphisms of cytokines genes (Ramos et al., 2012). The effect of the cytokine gene polymorphisms in the pathogenesis of HCV and HBV infections has additionally been explored (Pravica et al., 1999; Nelson et al., 2000; Lio et al., 2003; and Pestka et al., 2004).

IL6 is a multifunctional cytokine basic for the control of immune reaction (Kakumu et al., 1993). It exerts its actions by binding to its cell-surface receptor. (Galun et al., 2000). As of late, various reviews have detailed that polymorphisms close to the quality for IL-28B are emphatically associated with treatment reaction in patients with ceaseless hepatitis C and furthermore with unconstrained viral leeway (Di-Lulio et al., 2011 and Tanaka et al., 2009). Serum levels of IL6

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were observed to be altogether expanded in patients with chronic hepatitis B (Malaguarnera et al., 1997;) and in patients with chronic hepatitis C patients (Wang et al., 1999). However, the relationship between polymorphisms of IL6 gene with HCV and HBV progression has not been examined in Limpopo region. The present study aims to determine the impact of host genetic polymorphism at IL-6 gene on the occurrence of HBV and HCV in Limpopo province, South Africa.

1.3. Study hypothesis

In this study, we hypothesize that host genetic polymorphisms at IL-6 (-174 G/C) is associated with susceptibility to HCV and HBV.

High levels of IL-6 is associated with HCV and HBV.

1.4. General objective of the study

To determine the potential impact of hosts' genetic polymorphism on the occurrence of HCV and HBV among HIV/AIDS patients.

1.4.1. Specific objectives of the study

- To determine the sero-prevalence of HBV and HCV among HIV/AIDS patients in Limpopo province South Africa.
- To determine the distribution of IL-6 gene promoter (-174G/C) genetic polymorphisms
- To identify any association between and IL-6 genotypes and Hepatitis viral infection
- To evaluate the relationships between interleukin 6 serum levels in the serum with HBV and HCV.

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CHAPTER TWO

LITRATURE REVIEW

2.1. Interleukin-6

Interleukin 6 is known as the pleiotropic cytokine that modulates a variety of physiological events in vertebrates, e. g apoptosis, cell proliferation, differentiation and survival. It is an important cytokine that plays a role in the immune system, endocrine, inflammation, hematopoietic and the nervous system (Ara et al., 2010). There are variety of cells that are reported to produce IL-6. These include: T and B cells, eosinophils, macrophages, mast cells, fibroblast, smooth muscle cells, skeletal cells, keratinocytes, dendritic cells, chondrocytes and endothelial cells, these cell produce IL-6 only when there is an infection, trauma or injury (Ishihara et al., 2002).

2.1.1. Structure of IL-6 and the location of its gene in human chromosome

The amino acid sequence of human IL-6 contains about 212 amino acids of which 28 amino acids hydrophobic signal peptide is cleaved off resulting in a mature protein of 184 amino acids (Scheller et al., 2011). The molecular weight of this cytokine is recorded to be from 21KDa to 28KDa depending on post- transcriptional processing such as glycosylation and phosphorylation (May et al., 1988) with the isoelectric point at 5.4 (Sikka et al., 2013). It has a dimensional structure that is four- helix bundle (Cheng et al., 2013).

The human IL-6 gene, located in the short arm of the human chromosome 7p21, is approximately 5kb (Sehagal et al., 1986), with -174 G/C polymorphism in its promoter region. It consists of four

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introns and five exons. The human IL-6 gene contains three transcriptional initiation sites which then corresponds with three TATA- like sequences (Sehagal et al., 1986).

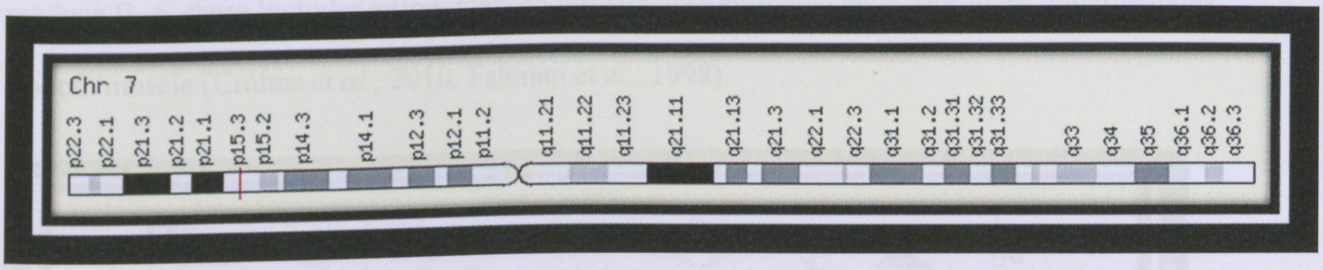


Figure 2.1: location of the gene coding for IL-6 on the chromosome: 7q; location: 7p15.3 (http://www.genecards.org/cgi-bin/carddisp.pl?gene=IL6).

2.1.2. Genomic location

This gene starts at 22.765.503bp and end at 22.771.621bp of the human gene. Alternative location of IL-6 gene is Chr7+CRA-TCAG 22.817.875-22.822.730 (Cheng et al., 2013).

2.1.3. Interleukin-6 Receptor

The receptor for IL-6 is known as gp80 or CD126 which contains an Ig-like domain and tandem fibronectin type III domains including a four -cysteine motif and a tryptophan-serine-tryptophan - serine motif in the extracellular region which are responsible for ligand binding, hence called cytokine-binding molecule (Hibi et al., 1996). CD126 receptor is found on hepatocytes, intestinal epithelial cells, endocrine glands such as pituitary and adrenal cortex and leukocytes, but not on naïve B cells or certain cell lines. In addition, IL-6R expression in osteoblast is unregulated by dexamethasone treatment (Ikeda et al., 2001). IL-6 has at least two types of soluble forms that are generated by proteolytic cleavage of the membrane-bound form of alternative splicing of its mRNA (Pennica et al., 1995).

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Interleukin 6 is produced by a variety of cell types such as monocytes, T cells, B cells, polymorphonuclear leukocytes, eosinophils, mast cells. The non-immune cells are also known to produce IL-6, these includes adipocytes, endothelial and epithelial cells, fibroblast, keratinocytes, smooth muscle (Crohns *et al.,* 2010, Fshman et al., 1998).

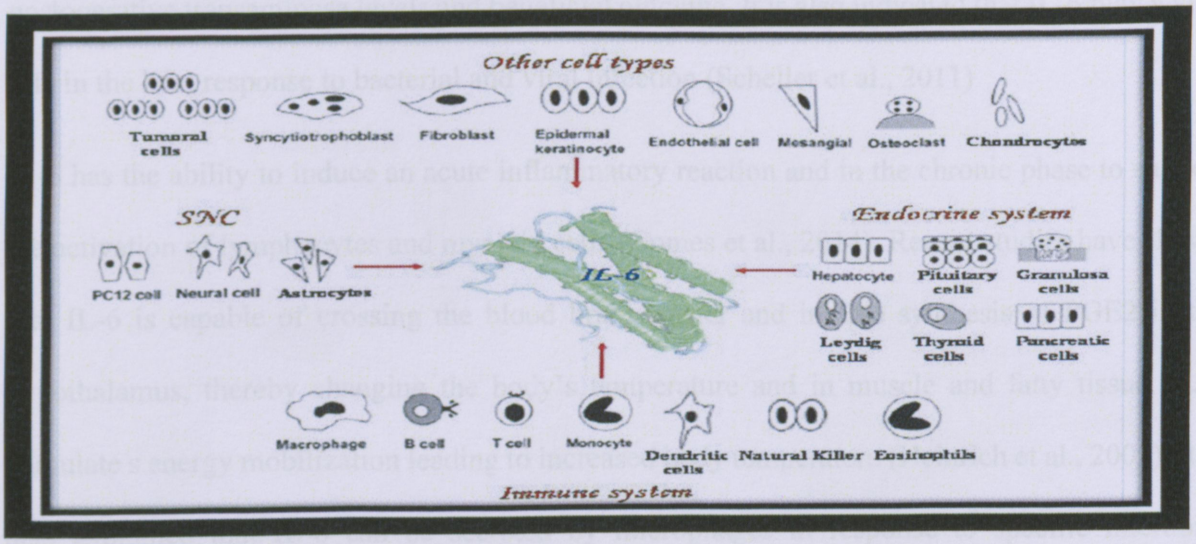


Figure 2.2: Diagram that shows the different types of cells that are reported to produce IL-6 (Estrada-Villasenor et al., 2007).

2.1.4. Function of IL-6

Interleukin -6, a member of the cytokine family, is described as a cytokine with many biological functions (Pine et al., 2011). It plays a role in metabolism, reproduction, immunology and neoplasm. It achieves its functions through its receptor, gp130. Il-6 plays a distinct role in acute phase inflammatory response by producing a variety of hepatic proteins called acute phase proteins (Sehgal et al., 1989). Il-6 stimulates proliferation of thymic and peripheral T cell (Nicola, 1994) and in cooperation with IL- 1 (Helle et al., 1988), it also induces T cell differentiation to cytolytic T cell and activates natural killer cells (Scheller et al., 2011).

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Interlukin-6 has been found to play a protogonic role in the defense against parasitic, bacterial and viral infections and also as being a major player during several autoimmune diseases, e.g., IL-6 determines the elimination of vaccinia virus and listeria monocytogenesis infections (Gabay., 2006). It has been defined that high IL-6 levels after liver resection in humans correlate with low postoperative transaminase levels and beneficial outcome. It is also indicated that IL-6 play a vital role in the host response to bacterial and viral infection (Scheller et al., 2011)

IL-6 has the ability to induce an acute inflammatory reaction and in the chronic phase to support the activation of lymphocytes and myeloid cells (Gomes et al., 2014). Recent studies have shown that IL-6 is capable of crossing the blood brain barrier and initiate synthesis of PGE2 in the hypothalamus, thereby changing the body's temperature and in muscle and fatty tissue, IL-6 stimulate s energy mobilization leading to increased body temperature (Heinrich et al., 2003). It is also explained that IL-6 can be secreted by microphages in response to specific microbial molecules known as pathogens associated molecular patterns (PAMP's) and these PAMP's bind to highly important group of detection molecules of the innate immune system called pattern recognition receptors and TLR's, which are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that gives rise to inflammatory cytolane production (Lin et al., 2007). It is shown that IL-6 is essential for hybridoma growth and is found in many supplemental cloning media such as briclone and the inhibitors of IL-6 including estrogen are used in the treatment of postmenopausal osteoporosis (Heinrich et al., 2003), it is also responsible for stimulating acute phase protein synthesis, as well as the production of neutrophils in the bone marrow and it supports the growth of B –cells and is antagonistic to regulatory T-cells (Scheller et al., 2011). IL- 6 is also involved in pathophysiology, it shows it functions in bone by stimulating osteoclast progenitor cell differentiation. It may also play an important role as a

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mediator of several infectious and autoimmune diseases, including HIV (Scheller at al., 2011 and Heinrich et al., 2003) and rheumatoid arthritis (Lin et al., 2007).

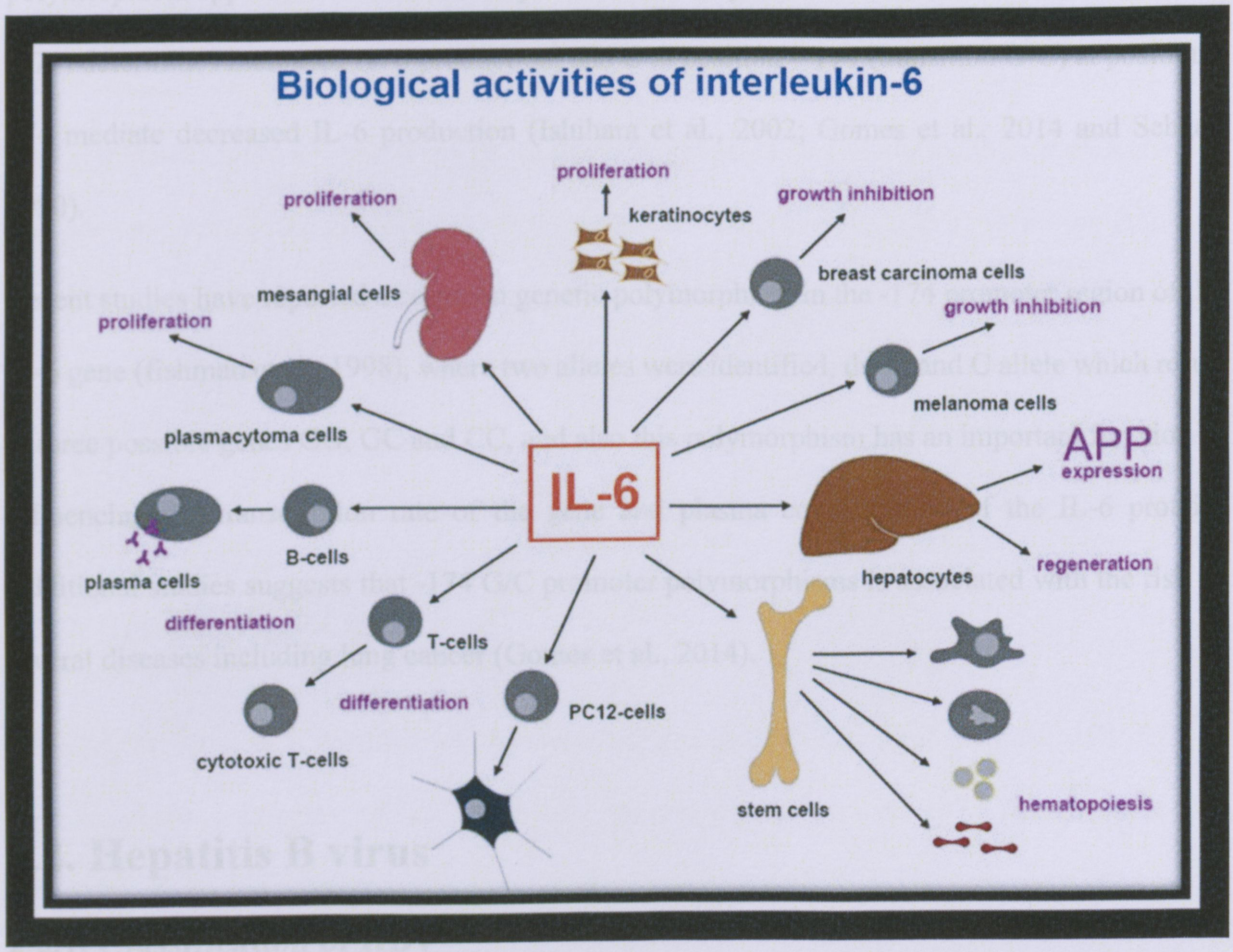


Figure 2.3: The figure shows some of the biological activities of IL-6 (Ishihara *et al.,* 2002).

2.1.5. IL-6 gene promoter polymorphisms

A polymorphism is a common, inherited variation in the DNA sequence. There are different types of IL-6 polymorphisms existing in the genome. The polymorphic allele vary between and within populations (Ara *et al.,* 2010).

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Common polymorphisms in pro-inflammatory and anti- inflammatory cytokines genes influence cytokine production and may play a role in diseases. There are four polymorphisms in the promoter region of IL-6 at position -597(G/A), -572(G/C),-373(A/G) and -174(G/C). The – 174 (G/C) polymorphism appears to influence IL-6 production: G at position -174 (transition C-G at position -174) determines increased IL-6 production, and C at position – 174 (transition G-C) at position -174 mediate decreased IL-6 production (Ishihara et al., 2002; Gomes et al., 2014 and Sehgal, 1990).

Recent studies have reported a common genetic polymorphism in the -174 promoter region of the IL-6 gene (fishman et al., 1998), where two alleles were identified, the G and C allele which result in three possible genes GG, GC and CC, and also this polymorphism has an important function in influencing the transcription rate of the gene and plasma concentration of the IL-6 protein. Additional studies suggests that -174 G/C promoter polymorphisms is associated with the risk of several diseases including lung cancer (Gomes et al., 2014).

2.2. Hepatitis B virus

2.2.1. Classification of HBV

The hepatitis B virus is classified as the type species of the *Orthohepadnavirus*, which contains three other species: the *Ground squirrel hepatitis virus*, *Woodchuck hepatitis virus*, and the *Woolly monkey hepatitis B virus*. The genus is classified as part of the *Hepadnaviridae* family, which contains two other genera, the *Avihepadnavirus* and a second which is yet to be assigned. This family of viruses have not been assigned to a viral order. Viruses similar to hepatitis B have been found in all apes (orangutan, gibbons, gorillas and chimpanzees), in Old World monkeys, and in

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a New World woolly monkeys suggesting an ancient origin for this virus in primates (Dupinay et al., 2013).

The virus is divided into four major serotypes (adr, adw, ayr, ayw) based on antigenic epitopes present on its envelope proteins. These serotypes are based on a common determinant (a) and two mutually exclusive determinant pairs (d/y and w/r). The viral strains have also been divided into ten genotypes (A–J) and forty subgenotypes according to overall nucleotide sequence variation of the genome (Hundie et al., 2016). The genotypes have a distinct geographical distribution and are used in tracing the evolution and transmission of the virus. Differences between genotypes affect the disease severity, course and likelihood of complications, and response to treatment and possibly vaccination (Kramvis et al., 2005). The serotypes and genotypes do not necessarily correspond. Genotype D has 10 sub-genotypes (Ghosh et al., 2013).

2.2.2. Morphology of HBV

Hepatitis B virus is a member of the Hepadnavirus family. The virus particle, called Dane particle (virion), consists of an outer lipid envelope and an icosahedral nucleocapsid core composed of protein (Zuckermann et al., 1996). The nucleocapsid encloses the viral DNA and a DNA polymerase that has reverse transcriptase activity similar to retroviruses (Locarnini, 2004). The outer envelope contains embedded proteins which are involved in viral binding of, and entry into, susceptible cells. The virus is one of the smallest enveloped animal viruses with a virion diameter of 42 nm, but pleomorphic forms exist, including filamentous and spherical bodies lacking a core (Locarnini, 2004). These particles are not infectious and are composed of the lipid and protein that forms part of the surface of the virion, which is called the surface antigen (HBsAg), and is produced in excess during the life cycle of the virus (Howard, 1986).

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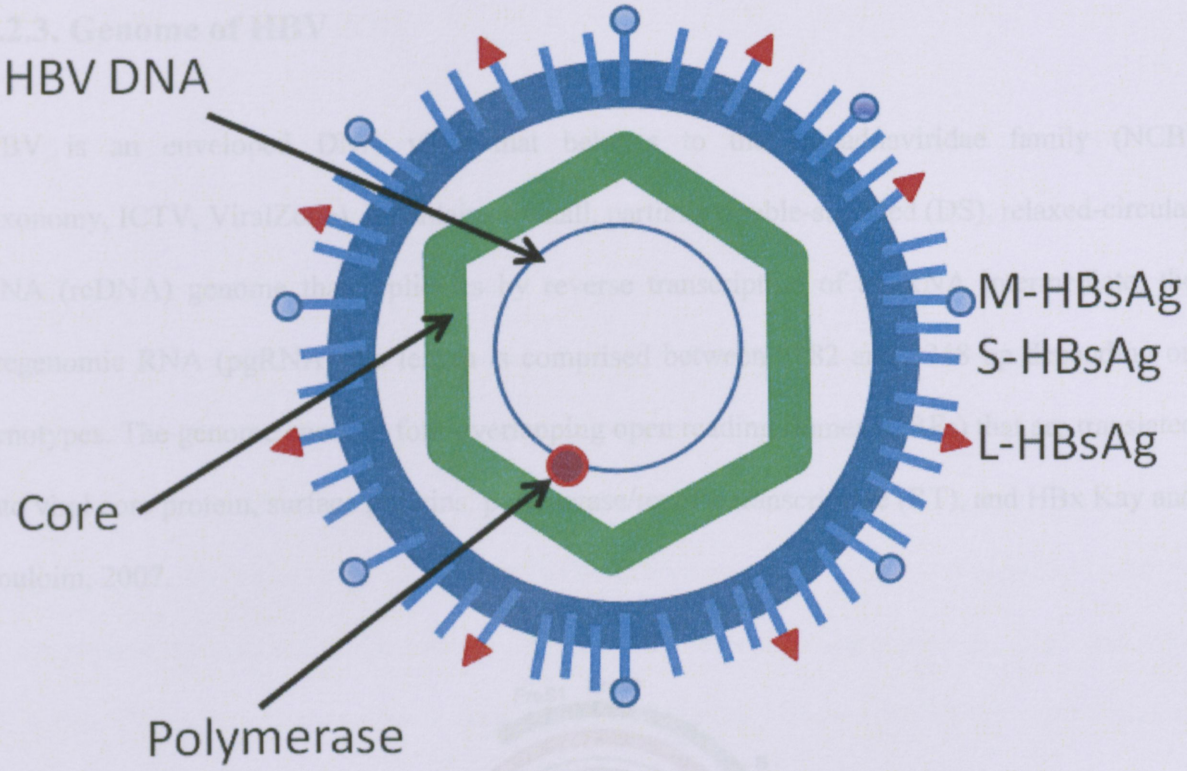


Figure 2.4: The HBV structure (Howard, 1986)

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2.2.3. Genome of HBV

HBV is an enveloped DNA virus that belongs to the Hepadnaviridae family (NCBI taxonomy, ICTV, ViralZone). It contains a small, partially double-stranded (DS), relaxed-circular DNA (rcDNA) genome that replicates by reverse transcription of an RNA intermediate, the pregenomic RNA (pgRNA). Its length is comprised between 3182 and 3248 bp depending on genotypes. The genome encodes four overlapping open reading frames (ORFs) that are translated into viral core protein, surface proteins, polymerase/reverse transcriptase (RT), and HBx Kay and Zouloim, 2007.

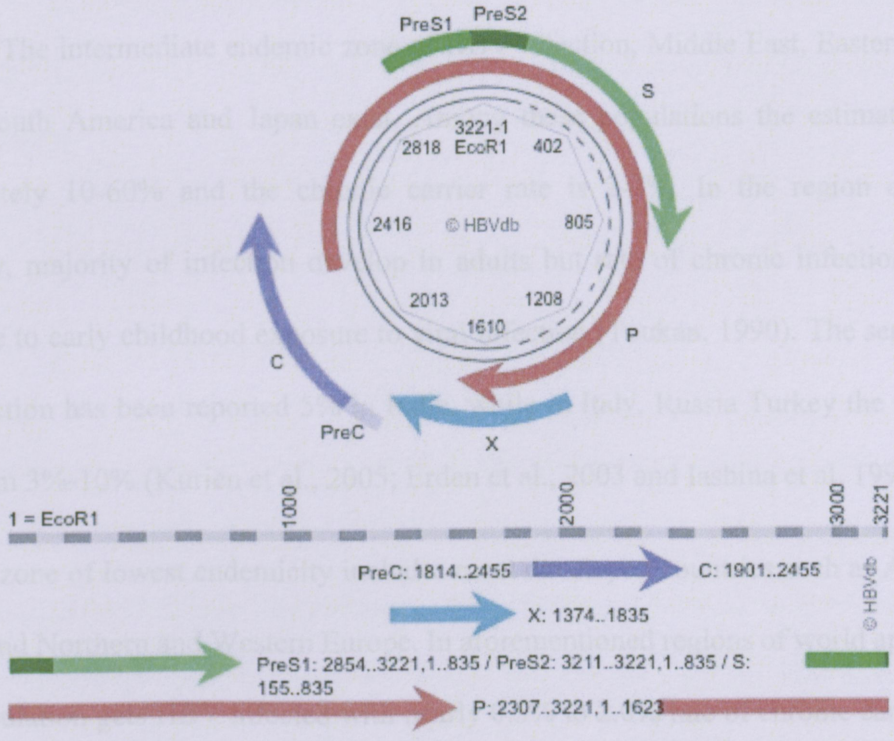


Figure 2.5: Genome of HBV (Kay and Zouloim, 2007)

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2.2.4. Epidemiology of HBV

The epidemiology of chronic HBV infection is distinct and diverse worldwide. Various sero-prevalence studies conducted in different areas of world can easily be categorized into three distinct groups of higher, intermediate and lower endemicity (Hou et al., 2005). In developing countries with larger population (South East Asia, Sub Sahara Africa, China, Indonesia, Nigeria and Amazon Basin), there is higher prevalence of endemicity with approximately 8% of population as chronic carrier of HBV. In aforementioned areas of world, 70% to 95% of population represents present or past serological markers against HBV. In another study, it has been reported that 60% of world population exist in high endemic zone of HBV infection (Margolis et al., 1991 and Alter M, 2003). The intermediate endemic zone of HBV infection, Middle East, Eastern and Southern Europe, South America and Japan exist. Among these populations the estimated infection is approximately 10-60% and the chronic carrier rate is 2-7%. In the region of intermediate endemicity, majority of infection develop in adults but rate of chronic infection are higher in infants due to early childhood exposure to viral infection (Toukan, 1990). The seroprevalence of HBV infection has been reported 5% in India, while in Italy, Russia Turkey the prevalence rate ranges from 3%-10% (Kurien et al., 2005; Erden et al., 2003 and Iashina et al. 1992).

The HBV zone of lowest endemicity includes most developed countries such as Australia, North America and Northern and Western Europe. In aforementioned regions of world approximately 5-7% of population gets HBV infected with nearly 0.5% to 2.0% rate of chronic carriers. The most probable reasons of HBV infection in young adolescents and could be exposure to high risk population groups, injection drug users, health care professionals, sex workers and unhealthy blood transfusion setups (Saeed et al., 2014).

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HBV infection is hyperendemic (> 8% of hepatitis B surface antigen (HBsAg) chronic carriers in the general population) only in some sub-Saharan countries such as Nigeria, Namibia, Gabon, Cameroon, Burkina Faso. Other countries like Kenya, Zambia, The Ivory Coast, Liberia, Sierra Leone and Senegal are considered areas of intermediate endemicity (2%-8%), while Egypt, Tunisia, Algeria and Morocco, located in the north of the continent, show a low endemicity level (< 2%) (Kramvis et al., 2007). Of note, the epidemiology in Africa is characterized by a much higher HBsAg prevalence in rural than in urban areas (Komas et al., 2013 and Peto et al. 2014) and by a greater risk for males of becoming HBV chronic carriers, with a male to female ratio ranging from 1.1:1 to 3:1 and increasing with the increase in age (Abebe et al., 2003; Uneke et al., 2005; Kirk et al., 2006). The higher percentage of HBsAg-positive males harboring HBV chronic infection may be the result of differences in tribal and sexual behaviors between males and females (Heikel J, 1999).

2.2.5. Life cycle and pathogenesis of HBV

After liver cell membrane attachment to most likely cell-associated heparan sulfate proteoglycans, the viral particle binds specifically to an unknown hepatocyte-specific preS1-receptor (Fig. 1) (Urban et al., 2010). Until now, the precise mechanism of viral entry has not been elucidated. Both, endocytosis and direct fusion of the viral envelope with the plasma membrane have been proposed as potential pathways. After uncoating/release into the cytoplasm and transport of the nucleocapsid to the nucleus, the partially double-stranded viral relaxed circular DNA (rcDNA) is repaired by both viral and cellular enzymes. Specifically, the incomplete plus-strand of the rcDNA is completed by the viral polymerase and in another step the viral polymerase and RNA-primers used

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for DNA plus-strand synthesis are removed by cellular enzymes (Nassal M, 2008). Eventually, covalently closed circular DNA (cccDNA) is formed by covalent ligation of both DNA strands. Lack of cccDNA formation in non-human host cells, such as hepatocytes from HBV transgenic mice suggests that host-specific factors may regulate this ligation process (Urban et al., 2010). There is evidence that each infected cell contains 1–50 cccDNA molecules as unique episomal minichromosomes. As a replicative intermediate of the HBV life cycle, cccDNA is crucial for the persistence of HBV infection. Importantly, this seems to be true even in patients that show serologic evidence of viral clearance (Balsono C and Alisi A, 2008). Viral cccDNA serves as a template for RNA synthesis. All viral RNA species are transcribed from the cccDNA using the cellular transcriptional machinery. The viral DNA contains four major open reading frames:

1. the precore/core gene, coding for the nucleocapsid protein and for the secreted, non-structural, precore protein, the HBeAg;
2. the polymerase gene coding for the reverse transcriptase, RNase H and the terminal protein domains;
3. the PreS1/L-, PreS2/M- and Surface/S-gene, coding for the three envelope proteins; and
4. the X gene, coding for the regulatory X-protein (Urban et al., 2010)

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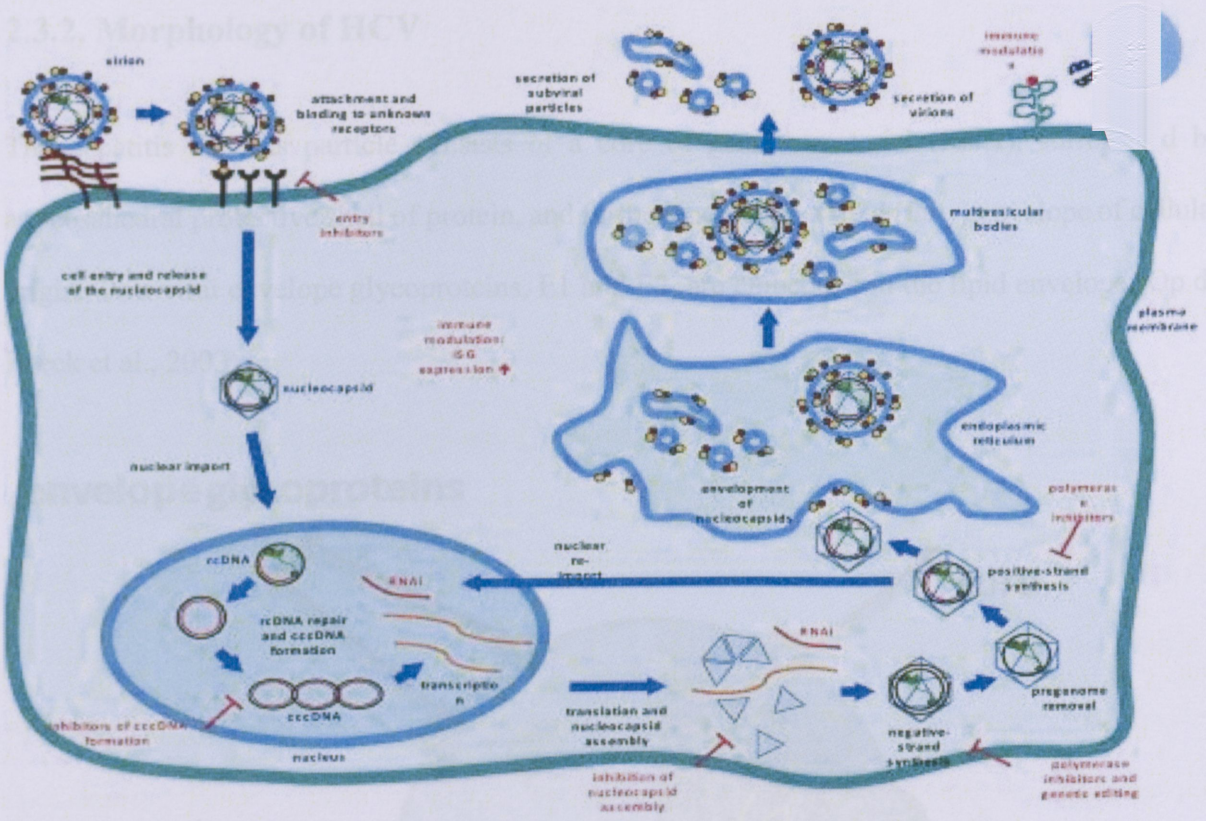


Figure 2.6: The structure showing the replication of HBV (Balsono C and Alisi A, 2008)

2.3. Hepatitis C virus

2.3.1. Classification of HCV

The hepatitis C virus belongs to the genus *Hepacivirus*, a member of the family *Flaviviridae*. Until recently it was considered to be the only member of this genus. However a member of this genus has been discovered in dogs-canine hepacivirus (Kapoor et al., 2011). There is also at least one virus in this genus that infects horses (Burbelo et al., 2012). Several additional viruses in the genus have been described in bats and rodents (Kapoor et al., 2013 and Quan et al., 2013).

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2.3.2. Morphology of HCV

The hepatitis C virus particle consists of a core of genetic material (RNA), surrounded by an icosahedral protective shell of protein, and further encased in a lipid (fatty) envelope of cellular origin. Two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope (Op de Beeck et al., 2003).

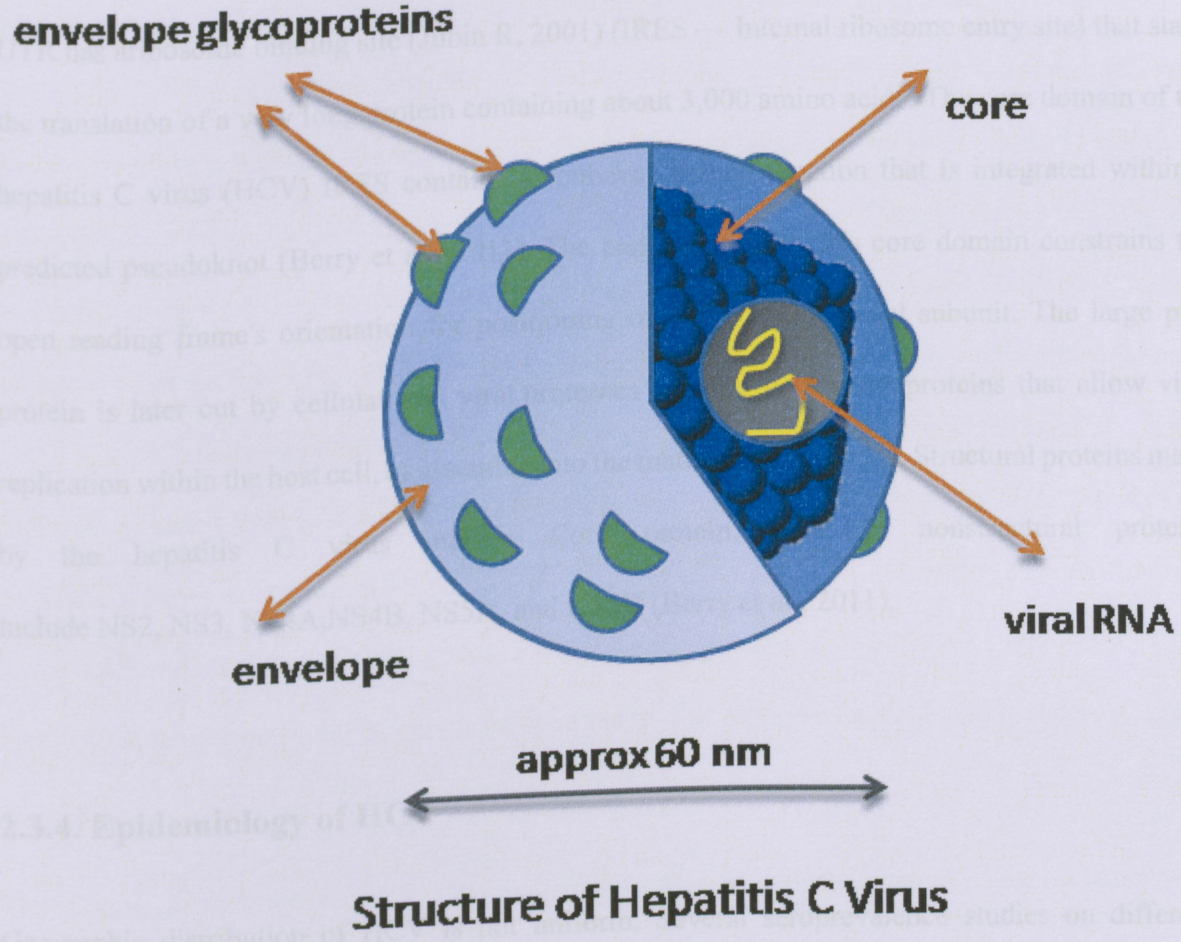


Figure 2.7: Structure of HCV virus (Op de Beeck et al., 2003)

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2.3.3. Genome of HCV

Hepatitis C virus has a positive sense single-stranded RNA genome. The genome consists of a single open reading frame that is 9600 nucleotide bases long (Kato N, 2000). This single open reading frame is translated to produce a single protein product, which is then further processed to produce smaller active proteins. At the 5' and 3' ends of the RNA are the UTR, which are not translated into proteins but are important to translation and replication of the viral RNA. The 5' UTR has aribosome binding site (Jubin R, 2001) (IRES — Internal ribosome entry site) that starts the translation of a very long protein containing about 3,000 amino acids. The core domain of the hepatitis C virus (HCV) IRES contains a four-way helical junction that is integrated within a predicted pseudoknot (Berry et al., 2011). The conformation of this core domain constrains the open reading frame's orientation for positioning on the 40S ribosomal subunit. The large pre-protein is later cut by cellular and viral proteases into the 10 smaller proteins that allow viral replication within the host cell, or assemble into the mature viral particles. Structural proteins made by the hepatitis C virus include Core protein, E1 and E2; nonstructural proteins include NS2, NS3, NS4A,NS4B, NS5A, and NS5B (Berry et al., 2011).

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2.3.4. Epidemiology of HCV

Geographic distribution of HCV is not uniform. Several seroprevalence studies on different populations tend to describe the epidemiological patterns of disease (Saeed et al., 2014). Although seroprevalence studies conducted on general population best describes the actual status of disease in that particular region, however majorities of such studies are conducted on high risk populations.

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Among various seroprevalence studies, majorities are based on cross sectional design; however population based studies are better representative of entire community. Limited prevalence studies have been documented in various regions of world due to expenses and practical difficulties involved in detection of viral RNA in serum. Available data suggests that HCV is prevalent in 3% of world population (Berry et al., 2011).

The prevalence of HCV is considered highest in African and Asian regions, but countries with limited prevalence include Northern and Western Europe, Australia and North America (Shepard et al., 2005). The GBD subdivides the African continent into 4 macro areas: Central, East, West and Southern, whereas the Saharan area (North Africa) is generally associated with Middle East countries. The estimated prevalence of anti-HCV in the whole Sub- Saharan Africa is 2.9% with an estimated 26.9 million of cases, ranging from 6.0% in the Central area and 0.9% in the Southern countries (Petruzziello et al., 2016). China is world's most populous country with approximately 1 347 million population with estimated 38 million people infected with HCV (Saeed et al., 2014). India is second world's most populous country with estimated population of 1 210 million population which covers approximately 17.25% of world population. According to a community-based study conducted in West Bengal, the estimated prevalence of HCV was reported to be of 0.9% (Populous countries wiki; prevalence of HCV was reported to be of 0.9%) (Chowdhury et al., 2003).

The highest prevalence of HCV infection (70%--90%) is reported among those persons with substantial or repeated direct percutaneous exposures to blood (e.g., IDUs, persons with hemophilia treated with clotting factor concentrates that did not undergo viral inactivation, and recipients of transfusions from HCV-positive donors). Moderate infection prevalence (10%) has been reported among long-term hemodialysis patients, and lower prevalence is reported among

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persons with high-risk sexual practices (5%) and health-care workers (1%--2%) (CDC, 2012). HCV is not transmitted efficiently through occupational exposure. The risk of acquiring HCV infection from a contaminated needle stick is <2%, and transmission rarely has been documented from mucous membrane or nonintact skin exposures (CDC, 2001).

2.3.5. Life cycle and pathogenicity of HCV

The life cycle of the virus is initiated when the virus attaches to the hepatocyte in the process of entry. Although the entire set of required entry factors is yet unknown, a number of necessary receptors and entry factors have been identified (Agnello et al., 1999). HCV entry is a multistep process and is initiated when cellular glycosaminoglycans and low density lipoproteins mediate attachment of the virion to the hepatocyte (Germi et al., 2003, and Molina et al. 2007). Next, E2 mediates binding to cellular receptor scavenger receptor class B type I (SR-BI), a lipoprotein receptor (Catanese et al., 2010; Scarselli et al., 2002 and Zeisel et al., 2007). The binding of E2 to SR-BI is believed to reveal a binding site on E2 which allows binding to cellular receptor CD81, a tetraspanin (Pileri et al., 1992 and Evans et al., 2007). The virus then is thought to translocate to the tight junctions where tight junction proteins claudin-1 (Evans et al., 2007) and occluding mediate entry. Recently, Niemann Pick C1-like 1 cholesterol absorption receptor (NPC1L1) has been shown to act as a late entry factor (Sainz et al., 2012). Additionally, cellular kinases epidermal growth factor and ephrin receptor A2 were recently reported as entry cofactors (Lupberger et al., 2011). Hence, HCV entry is a complex, multistep process. The virion is then taken up by the cell by a clathrin-mediated, pH-dependent, endocytic process (Blanchard et al., 2006). Upon acidification of the endosome, the viral envelope fuses with the endosomal membrane triggering the uncoating and release of the viral genome is deposited in the host cell cytoplasm (Tscheme et

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al., 2006). The RNA genome initially serves as template for translation of the viral polyprotein by the host cell translational machinery. This polyprotein is co- and post-translationally cleaved by host and viral proteases into at least 10 individual gene products. Host signal peptidases cleave the structural proteins (core, E1, E2, and p7) while two viral proteases (NS2 and NS3 along with cofactor NS4A) cleave the nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The viral polymerase NS5B then uses the viral positive-strand genome as template to generate negative-strand HCV RNA intermediates, which are subsequently used as a template to make more of the positive-strand RNA genome. HCV RNA replication takes place on a specialized structure in the cytoplasm of the cell known as the "membranous web." This structure is induced by HCV infection and NS4B expression alone is sufficient for the reorganization of ER-derived cellular membranes into membranous webs (Egger et al., 2002). The multi-functional genomic RNA is then packaged within viral capsids, which are thought to become enveloped as they bud into the endoplasmic reticulum and are released from the cell through the secretory pathway (Gosert et al., 2003).

2.4. Transmission of Hepatitis virus infection

The hepatitis B virus is transmitted through blood and bodily fluids. This can most commonly occur in the following ways: direct contact with infected person. Children acquire HBV infection most frequently by parenteral horizontal transmission from parents (Kramavis et al., 2005). Unsafe sharing in the daily practices of toiletries and sharpening, cutting, scraping or scratching objects accounts for such a high horizontal transmission. In addition, cultural practices like scarification and tattooing and promiscuous sexual activity greatly increase the risk of HBV infection (Saeed et

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al., 2014). HBV transmission through the transfusion of blood or blood products still occurs (Kramavis et al., 2005).

2.6. Prevention, treatment and control of hepatitis virus

Safe and effective hepatitis B vaccines containing inactivated HBsAg have been available since the early 1980s. The first vaccines were plasma derived; however, these have been replaced over the years by vaccines manufactured in yeast or mammalian cells using recombinant DNA technology (Shouval D, 2003). In general, the vaccine is administered using a three-dose schedule. Vaccine efficacy (defined as antiHBs concentration of $10 mIU/ml) is greatest in infants, children and young adults with protective achieved in 95% of those vaccinated. After the age of 40 years, the proportion of persons who have a protective antibody response following vaccination declines to, 90% and to 75% in those vaccinated over the age of 60 years (Nguyen et al., 2009). Other factors associated with a reduced response to vaccination include immunosuppression, liver disease, renal failure, smoking and obesity (Rong et al., 2010). Protection conferred by hepatitis B vaccination has been shown to be long lasting, with the risk of HBV infection significantly reduced even when anti-HBs concentrations decline to 10 mIU/ml over time (Aspinall et al., 2011). Smoking and excessive alcohol consumption are associated with a poorer prognosis in chronic hepatitis virus infection, and patients should be offered lifestyle advice accordingly. Patients should be advised on the prevention of other blood-borne viruses, and vaccination against hepatitis A should be offered to those not already protected as a result of previous immunization or infection (Aspinall et al., 2011).

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2.7. Immune response to HBV

As with most infections, the immune system plays an elementary role in the body's attempts to eradicate the infection (Fong et al., 1994). The initial and most important immune response to HBV infection is local, where the immune cells such as leukocytes are recruited to the site of infections. The innate immune system is activated by pathogens by using pattern-recognition receptors (PRRs) that recognize specific structures on pathogens, such as double-stranded RNA and bacterial wall components. The most important PRRs in viral infections are Toll-like receptors (TLRs), RNA helicases, such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5), and double stranded RNA-dependent protein kinase (PKR) (Busca and Ashok, 2014).

Earlier studies have evaluated whether HBV has the ability to stimulate the interferon pathway of the innate immune response during the early stage of infection. Data from patients and animal models have shown that there is a delay between HBV inoculation and efficient replication (Fong et al., 1993 and Guidotti et al. 1999). Cytokines have also been reported to control HBV replication in the transgenic mouse model, including interleukin 12 (IL-12) (Cavanaugh et al., 1997) and IL-18 (Kimura et al., 2002), the effect being mediated by induction of IFN-γ and IFNα/β respectively. Injection of HBV transgenic mice with CD40 ligand to induce maturation of antigen presenting cells lead to recruitment of macrophages, dendritic cells and NK cells to the liver and induced secretion of TNF-α, IFN-γ, and IFNα/β, which ultimately resulted in inhibition of HBV replication (Kimura et al., 2002). In a primary human hepatocyte model, Rauch., (2010) showed that briefly after infection, liver macrophages recognize HBV and induce production of TNF-α, IL-6, IL-8 and IL-1β. However, the ability to control viral replication was attributed to IL-6 via NF-κB activation (Rauch et al., 2010).

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CHAPTER THREE:

MATERIALS AND METHODS

3.1. Ethical Clearance

The study was approved by the Health and Ethics committee of the University of Venda and the Limpopo department of health, in Polokwane. The objectives of the study were clearly explained to the patients, and those who agreed were asked to sign an informed consent form. Informed consent was obtained from all the study participants prior to enrolment in the research, and the human experimentation guidelines were followed.

3.2. Study site

The study was undertaken in different clinics and hospitals around three major districts of Limpopo Province namely: Waterberg, Sekhukhune and Capricorn Districts. The districts are characterized and dominated mostly by rural and semi urban areas were the distribution of facilities is poor or almost nonexistent.

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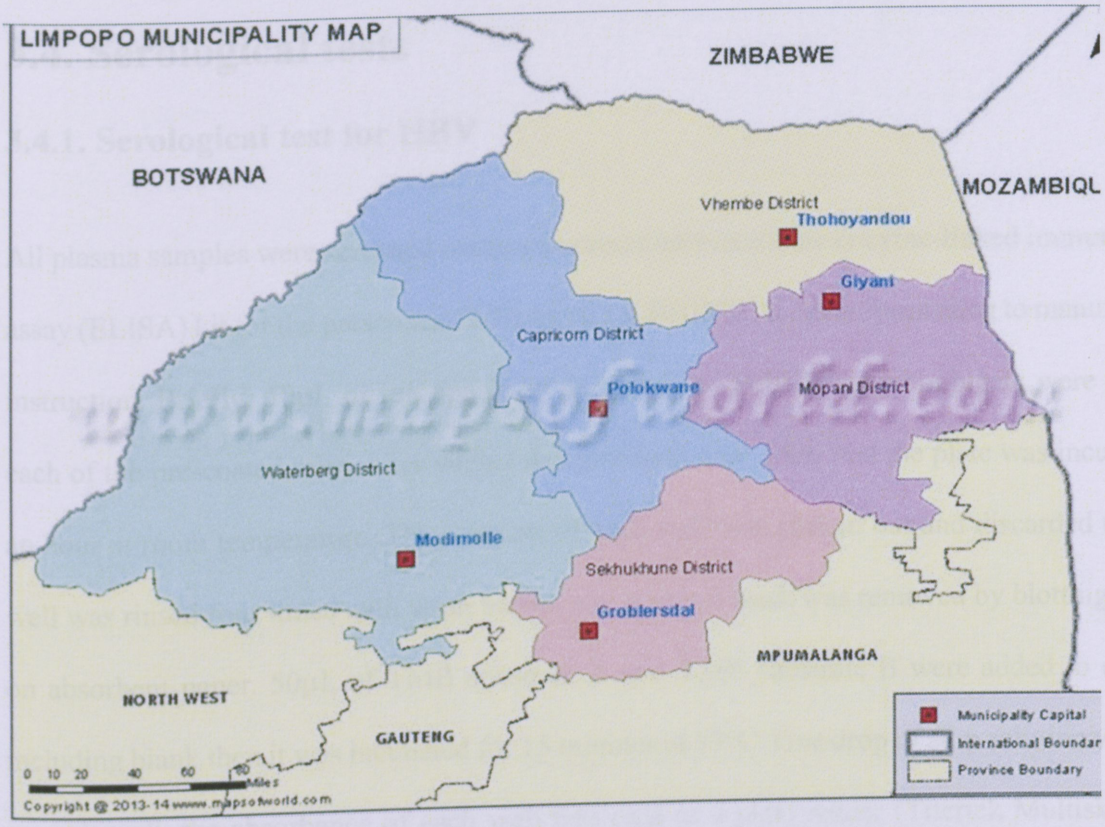


Figure 3.1: Map showing areas and districts of Limpopo Province (www.mapAfrica.com)

3.3. Sample collection and processing

A total of 466 blood samples were collected at Polokwane hospital in Limpopo province using ETDA tubes, and transported in cooler boxes to the laboratory of Microbiology at the University of Venda. All samples were properly labelled with patient's code, and date of collection, age, gender, HIV status and CD4 cell count. Upon arrival the samples were centrifuged for 10 minutes at 5000 rmp, then separated into plasma, buffy coat and red blood cells. The different components of the samples were stored at -20°C until further analysis.

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3.4. Serological tests

3.4.1. Serological test for HBV

All plasma samples were screened using a commercially available enzyme-linked immunosorbent assay (ELISA) kit for the presence of HBsAg (CTK BIOTECH, USA) according to manufacturer's instruction. Briefly, 50µL of test specimen and 50µL of HRP-HBsAb conjugate were placed in each of the pre-coated micro titer wells (also provided with a kit) and the plate was incubated for an hour at room temperature. The contents of each well was shaken out and discarded then each well was rinsed four times with wash buffer, any residual wash was removed by blotting the plate on absorbent paper. 50µL of TMB substrate A and TMB substrate B were added to each well including blank then it was incubated for 15 minutes at 37°C. One drop of stop solution was added to each well, the absorbance of each well was read in a plate reader (Titertek Multiskan; Flow Laboratories, McLean, VA), at 450nm (with the reading at 620nm used as reference).

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3.4.2. Serological test for HCV

All plasma samples were screened using a commercially available enzyme-linked immunosorbent assay (ELISA) for the presence of HCV Ab (CTK BIOTECH, USA) according to manufacturer's instructions. Briefly, 110 µL of diluted specimen and controls were placed in each of the pre-coated micro titer wells (provided with a kit) and the plate was incubated for 30 minutes at 37°C. The contents of each well was shaken out and discarded then each well was rinsed four times with wash buffer, any residual wash was removed by blotting the plate on absorbent paper. Two drops (100 µL) of HRP- anti-protein A conjugate was added into each well and it was incubated at 37°C for 30 minutes then the contents of each well was shaken out and discarded then each well was

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rinsed four times with wash buffer, any residual wash was removed by blotting the plate on absorbent paper. 50µL of TMB substrate A and TMB substrate B was added to each well respectively and it was incubated for 10 minutes at 37°C. One drop of stop solution was added to each well, the absorbance of each well was read in a plate reader (Titertek Multiskan; Flow Laboratories, McLean, VA), at 450nm (with the reading at 620nm used as reference).

3.4.3. Measurement of IL-6 concentration in the plasma samples

The concentration of IL-6 in the plasma samples was determined by using an ELISA kit (MABTECH, Australia, Pty Ltd) according to manufacturer's instruction. Briefly, high protein binding ELISA plates were coated with mAb 13A5, diluted to 0.5 µg/ml in PBS at pH 7.4, and 100 µl/well was added then incubated overnight at 4-8°C, the plate was washed twice with PBS (200µl). IL-6 standard was prepared by reconstituting contents of vial 4 in 1ml PBS to a concentration of 1 µg/ml. The stock solution was diluted to provide the standards as per guideline from the manufacturer and 100 µl/well of sample or standards were added to the plates and was incubated for 2 hours at room temperature. The plate was washed 5 times with PBS containing 0.05% Tween and 100 µl/well of the first conjugate (mAb 39C3-biotin) at 1 µl/ml was added to the plate and incubated for an hour at room temperature. Following incubation, the plate was rinsed 5 times with PBS containing 0.05% Tween. 100 µl/well of Streptavidin-HRP diluted 1:1000 was added and was incubated for an hour at room temperature then the plate was rinsed 5 times with PBS containing 0.05% Tween. After washing, 100 µl/well of substrate solution was added to the plate and the optical density was measured using ELISA reader.

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3.5. Genomic DNA extraction

DNA was extracted from the buffy coat (white blood cells) using GenElute blood Genomic DNA kit from Sigma Aldrich according to manufacturer's instruction. Briefly, 20 µL of proteinase k solution was placed into a 2 mL micro centrifuge tube and 200 µL of whole blood sample was added, then lysis solution C was added then vortexed for 15 minutes and incubated at 55°C for 10 minutes. The 500µl of the column preparation solution was added to each pre-assembled GenElute Miniprep Binding Column (with a red O-ring) and centrifuged at 12,000 5 *g* for 1 minute. The flow-through liquid was discarded. About 200 µl of ethanol (99.9%) was added and the entire contents was transferred into the treated column (pre-assembled GenElute Miniprep Binding column) and centrifuged at 6500 ×g for 1 minute. The tube containing the flow-through liquid was discarded and the column was placed in a new 2 mL collecting tube. 500 µL of prewash solution was added into column and centrifuged at 6500 ×g for 1 minute then 500 µL of wash solution was added and centrifuged at maximum speed (14000 ×g) for 3 minutes and the flow through liquid was discarded and the column was placed in a new 2mL collecting tubes. 200 µL of elution solution was directly placed into the center of the column and centrifuged at 6500 ×g for 1 minute to elute the DNA. After elution, the DNA was stored at -20°C for further analysis.

3.6. Amplification of IL-6 -174G/C

The extracted DNA were genotyped for IL-6 promoter gene at -174G/C polymorphism by Polymerase Chain Reaction restriction fragment length polymorphism (PCR-RFLP) as previously described by Ghazouani et al., (2011). The primers 5'-GGAGTCACACACTCCACCT-3' (IL6-sense) and 5'-CTGATTGGAAACCTTATTAAG-3' (IL6-antisense) were used. Briefly, 25.0 µL volume PCR was prepared which consisted of 5.0 µL of DNA, 12.5 µL Dream Taq (Inqaba

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Biotech, Pretoria, South Africa), 0.6 µL IL-6 -174 primer (both forward and reverse), 0.25 µL BSA and 6.05 µL nuclease free water. The PCR condition were as follows: the initial denaturation at 95°C for 5 min was followed by 40 cycles of 94°C for 30 s, 56°C annealing for 40 s, extension at 72 °C for 30 s and final extension at 72°C for 7 minutes. The amplified products were digested overnight with 5 units of the restriction enzyme NlaIII at 37 °C and analyzed by electrophoresis in a 2.5% agarose gel stained with ethidium bromide.

3.6. Sequence analysis

The PCR products of some amplicons were sent for sequencing at Inqaba Technologies (Inqaba, Pretoria, South Africa). Sequences were edited using Staden package. Thereafter, bioedit package was used to align sequences which were then aligned using the Clustal W multiple aligner. With this package the nucleotides for all genes were aligned alongside a referral strain from the Genebank to determine the similarity and differences between the gene products from different samples.

3.7. Statistical analysis

The results were entered on an excel spread sheet and edited appropriately and analyzed using Statistical Package for social sciences (SPSS version 17.0). Data was summarized using frequency tables and bar charts. Contingency tables were used and the strength of association was measured using the chi-square and its associated p-value. The results were considered to be statistically significant when the p-value obtained is less than 0.05.

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CHAPTER FOUR:

RESULTS

4.1 General characteristics of the study participants

Most of the study participants were females (56.4). Majority of our study participants were aged between 21 to 45 years (52.4%). About 29.8% of our study participants had a CD4 cell count between 251-500. Most of the study participants were from Capricorn district (38.2%). Sekhukhune district had 19.7% while Waterberg had the least participants (17.0%). Most of the samples received originated from clinics (49.8%) while only 29.2% were from hospital settings. The results are shown on table 4.1 below.

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Table 4.1 Demographic characteristics of the study population

Characteristics		Frequency	Percentage (%)
Gender	Male	107	23.0
	Female	263	56.4
	Unknown	96	20.6
Age group	0-20 years	37	7.9
	21-45 years	244	52.4
	>46 years	75	16.1
	Unknown	110	23.6
CD4 count	0-250	93	20.0
	251-500	139	29.8
	501-1000	108	23.2
	>1001	17	3.6
	Unknown	109	23.4
District	Capricorn	178	38.2
	Sekhukhune	92	19.7
	Waterberg	79	17.0
	Unknown	117	25.1
Origin	Clinic	232	49.8
	Hospital	136	29.2
	Unknown	98	21.0

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4.2. Distribution of HCV and HBV in the study population

Out of the 455 samples tested for anti-HCV antibodies, 15.4% were positive, while for HBsAg 20.9% were positive. The results are shown as illustrated in **figure 4.1** below.

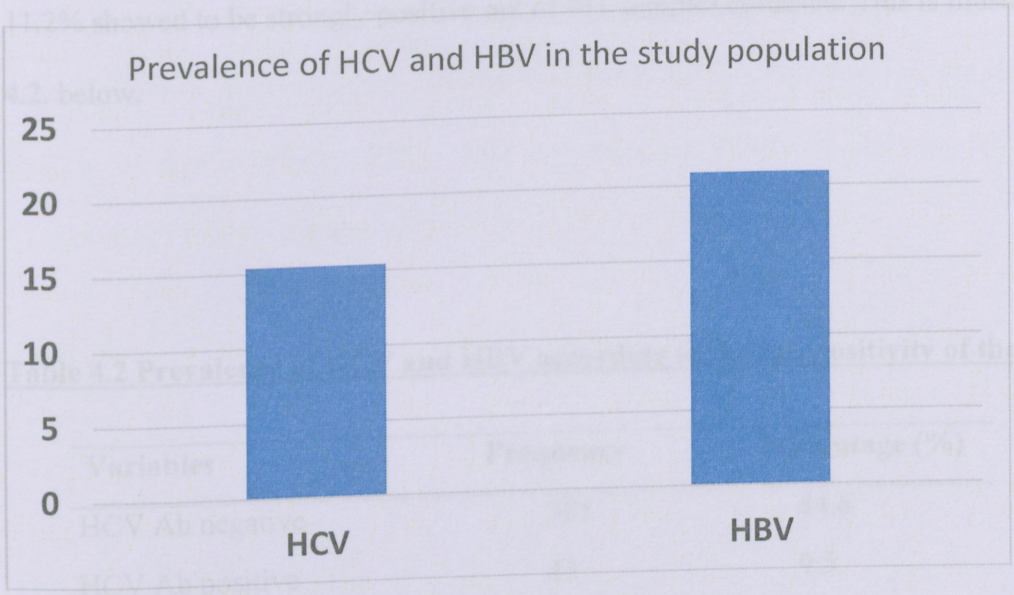


Figure 4.1: Frequency of occurrence of HCV and HBV among HIV patients.

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4.3. Prevalence of HCV and HBV according to the seropositivity of the test samples

Based on the OD values obtained, the samples were classified as negative, positive and strong positive. Out of the 455 samples tested for HCV Ab, 5.9% showed strong positivity. For HBs Ag 11.2% showed to be strongly positive out of 411 samples collected. This is illustrated on **table 4.2.** below.

Table 4.2 Prevalence of HCV and HBV according to the seropositivity of the test samples

Variables	Frequency	Percentage (%)
HCV Ab negative	385	84.6
HCV Ab positive	43	9.5
HCV Ab strong positive	27	5.9
Total	455	100.0
HBsAg Negative	325	79.1
HBsAg positive	40	9.7
HBsAg strong positive	46	11.2
Total	411	100

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4.4. Distribution of HCV Ab based on gender, age, CD4 cell count, District and Origin

The results obtained show that for the infection with HCV, males (15.1%) were more infected compared to females (13.2%) although there was no statistically significant difference (p=0.629). The highest rate of infection was among participants who were 45 years and older (20.8%) and the lowest rate was amongst those who are 0-20 years old (5.4%), However the difference was not statistically significant (p=0.076). There was not much difference observed while looking at the CD4 count of the participants (p=0.868).

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Table 4.3 Distribution of HCV based on gender, age, cd4 count, district and origin

Characteristics		HCV Ab	Total	Statistics
Gender	Male	16(15.1%)	106	χ^2=0.233, p=0.629
	Female	34(13.2%)	224	
	Unknown	20(20.8%)	96	
Age group	0 – 20 years	2(5.4%)	37	χ^2=5.167, p=0.076
	21-45 years	32(13.3%)	241	
	>45 years	15(20.8%)	72	
	Unknown	21(19.1%)	110	
CD4 count	0-250	17(18.5%)	92	χ^2=3.010, p=0.390
	251-500	17(12.5%)	136	
	501-1000	13(12.3%)	106	
	>1001	1(5.9%)	17	
	Unknown	22(20.2%)	109	
District	Capricorn	22(12.6%)	175	χ^2=0.446 p=0.800
	Sekhukhune	13(14.3%)	91	
	Waterberg	12(15.6%)	77	
	Unknown	23(19.7)	117	
Origin	Clinic	28(12.2%)	229	χ^2=1.315 p=0.251
	Hospital	22(16.5%)	133	
	Unknown	20(20.4%)	98	

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4.5. Distribution of HBV based on gender, age, CD4 count, district and origin

Table 4.4 illustrates the distribution of HBV by gender, age, CD4 count, district and origin. There was not much difference in terms of gender however males were mostly infected (22.0%) than females (21.1%) and the results did not show any statistical significance (p=0.858). Based on the age groups the highest infection rate was found within the participants above 45 years with 21.4% as compared to 21.1% in those who were 0 to 20 years, and above, the results did not show any statistical significance (p=0.076). Participants with a CD4 count above 1001 were mostly infected (33.3%) and the results did not show any statistical significance (p=0.675). Waterberg district had the lowest rate of infections (12.9%) compared to Sekhukhune district (27.1%) and Capricorn district (22.5%). Participants who were from hospital had higher (23.4%) than those in clinic (20.4%) (p=0.521).

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Table 4.4 Distribution of HBV based on gender, age, cd4 count, district and origin

Characteristics		HBV Ab	Total	Statistics
Gender	Male	22(22.0%)	100	χ^2=0.032, p=0.858
	Female	49(21.1%)	2320	
	Unknown	15(27.3%)	55	
Age group	0 – 20 years	7(21.2%)	33	χ^2=5.167, p=0.076
	21-45 years	45(20.6%)	218	
	>45 years	15(21.4%)	70	
	Unknown	19(17.2%)	110	
CD4 count	0-250	17(21.0%)	81	χ^2=4.708, p=0.675
	251-500	25(19.8%)	126	
	501-1000	22(22.7%)	97	
	>1001	5(33.3%)	15	
	Unknown	17(15.9%)	107	
District	Capricorn	35(22.5%)	158	χ^2=0.446 p=0.800
	Sekhukhune	23(27.1%)	85	
	Waterberg	9(12.9%)	70	
	Unknown	23(19.7)	117	
Origin	Clinic	42(20.4%)	206	χ^2=0.412 p=0.521
	Hospital	29(23.4%)	124	
	Unknown	15(15.3%)	98	

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4.6. Genotyping of IL-6 Single Nucleotide Polymorphisms (174G/C del polymorphism)

RFLP-PCR method was employed to genotype IL-6 from human samples. All samples amplified for interleukin-6. Figure 6 shows undigested PCR product and Figure 4.2 shows digested PCR products.

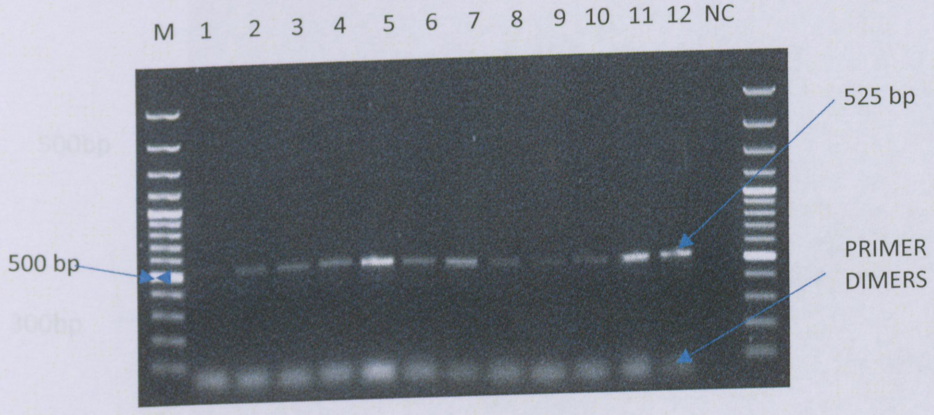


Figure 4.2: A representative 3% agarose gel photograph of IL-6 174 polymorphism stained with ethidium bromide before digestion.

M-Marker; Lane 1-12-Samples; NC-Negative Control.

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PCR product was digested by restriction enzyme (NlaIII) and visualized on 1% agarose gel, Figure 4.3. M represents the molecular weight marker (100bp), NC-negative control, Lane 1-17 test samples. Lane 1, 10, 11, 14, and 17 represents GG genotype; lane 2, 3, 4, 5, 8 9 represents CC genotype and lastly lane 12, 13 and 16 represent GC genotype.

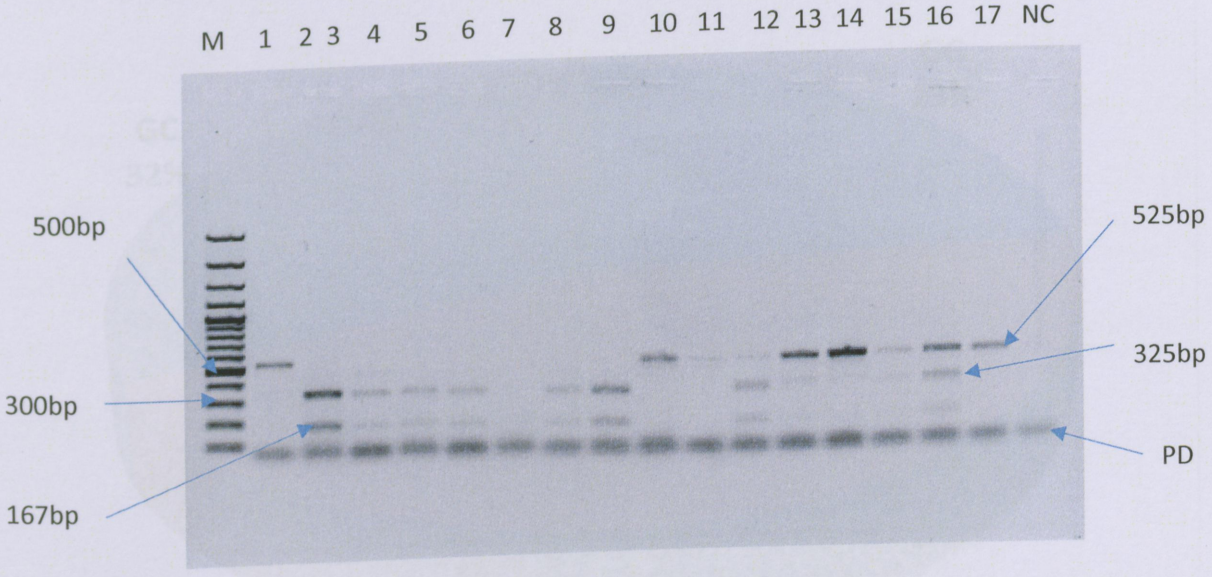


Figure 4.3: A representative 1% agarose gel photograph of IL-6-174G>C polymorphism stained with ethidium bromide after digestion.

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4.7. Distribution of IL-6 genotypes in the study population

Distribution of the different IL-6 genotypes where (45%) CC, 32% GC, and 23% GG showed on **figure 4.2**

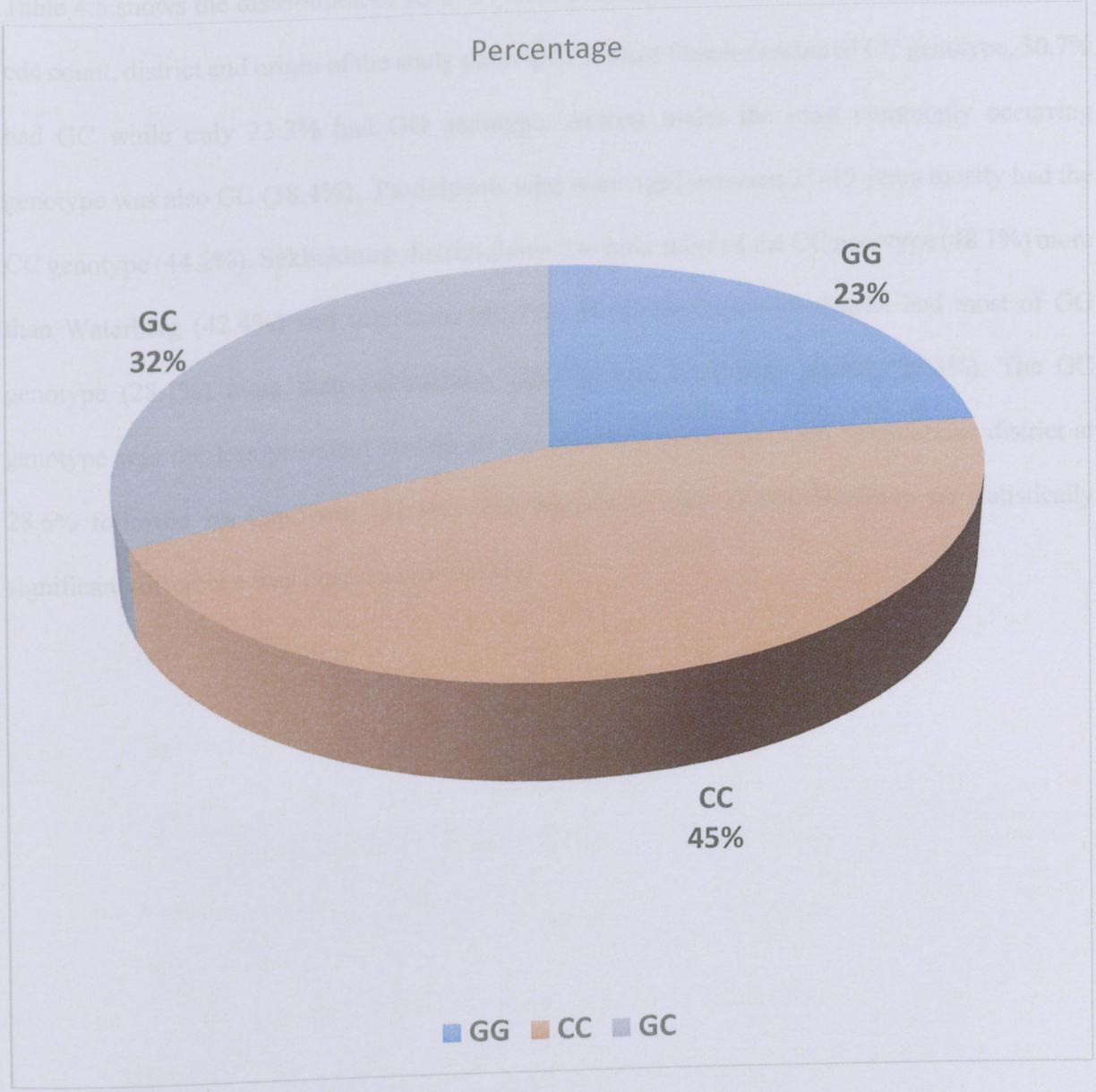


Figure 4.4 Overall distribution of IL-6 genotypes in the study population.

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4.7. Distribution of IL-6 genotypes according to gender, age group and cd4 count of the study participants.

Table 4.5 shows the distribution of IL-6 -174G/C polymorphism according to gender, age group, cd4 count, district and origin of the study participants. Most females harbored CC genotype, 30.7% had GC while only 23.3% had GG genotype. Among males the most commonly occurring genotype was also CC (38.4%). Participants who were aged between 21-45 years mostly had the CC genotype (44.2%). Sekhukhune district showed to have most of the CC genotype (48.1%) more than Waterberg (42.4%) and Capricorn (40.7%), However Capricorn district had most of GG genotype (28.1%) more than Sekhukhune (23.4%) and Waterberg district (20.3%). The GC genotype was the less prevalent among all the observed genotypes with Sekhukhune district at 28.6% followed by Capricorn (31.1%) and Waterberg with 37.3%, However no statistically significant difference was observed (p=0.631).

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Table 4.5: Distribution of IL-6 genotypes according to gender, age group, CD4 cell count, District and origin of the study participants

Characteristics		IL-6 genotypes			Statistics
		GG	CC	GC	
Sex	Male	24(27.9%)	33(38.4%)	29(33.7%)	χ^2=1.512, p=0.469
	Female	47(23.3%)	93(46.0%)	62(30.7%)	
Age Group	0-20 years	11(35.5%)	15(48.4%)	5(16.1%)	χ^2=4.538, p=0.338
	21-45 years	46(24.2%)	84(44.2%)	60(31.6%)	
	> 45 years	13(22.4%)	24(41.4%)	21(36.2%)	
CD4 Count	0-250	13(18.8%)	32(46.4%)	24(34.8%)	χ^2=4.947, p=0.551
	251-500	23(21.3%)	48(44.4%)	37(34.3%)	
	501-1000	26(30.6%)	33(38.8%)	26(30.6%)	
	>1001	5(31.3%)	8(50.0%)	3(18.8%)	
District	Capricorn	38(28.1%)	55(40.7%)	42(31.1%)	χ^2=2.575, p=0.631
	Sekhukhune	18(23.4%	37(48.1)	22(28.6%)	
	Waterberg	12(20.3%)	25(42.4%)	22(37.3%)	
Origin	Clinic	42(23.5%)	80(44.7%)	57(31.8%)	χ^2=0.457 p=0.796
	Hospital	29(26.9%)	45(41.7%)	34(31.5%)	

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4.8. Association of HCV and HBV with IL-6 genotypes

The samples which were positive for HCV had most of the GC genotype (20.9%), GG (13.6%) and CC (13.1%) respectively although the results did not show any statistical significance (p=0.189). For participants who were positive for HBV the most commonly occurring genotype was found to be GC (26.4%), CC (25.5%) and GG (21.1%) respectively, however the difference was not statistically significant (p=0.684). The data has been illustrated on table 4.6 below.

Table 4.6: Association of HCV and HBV with IL-6 genotypes

Variables		IL-6 Genotype			Statistics
		GG	CC	GC	
HCV	HCV negative	70(86.4%)	139(86.9%)	87(79.1%)	χ^2=3.337, p=0.189
	HCV positive	11(13.6%)	21(13.1%)	23(20.9%)	
HBV	HBV negative	60(78.9%)	114(74.5%)	78(73.6%)	χ^2=0.760, p=0.684
	HBV positive	16(21.1%)	39(25.5%)	28(26.4%)	

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4.9. Association of IL-6 -174G>C polymorphism and HCV patients

Genotyping of IL-6 -174G>C polymorphism in both patients and healthy controls was done by PCR-RFLP. The genetic models between patients and healthy controls. GC genotype showed highest percentage in the HCV positive participants in the over-dominant genetic model even though the results was not statistically significant (p=0.058; OD=0.567 and CI=0.314-1.025). The recessive genetic model was dominated by CC+GC (p=0.529; OD=0.796 and CI=0.390-1.069).

Table 4.7: Association of IL-6 genetic models with HCV

Models		healthy	HCV	P value	Odd ratio(95% CI)
Over dominant model	GC	86 (29.0%)	23 (41.8%)	0.058	0.567 0.314-1.025
	CC + GG	211 (71.0%)	32 (58.2%)		
Recessive model	CC + GC	226 (76.1%)	44 (80.0%)	0.529	0.796 0.390-1.069
	GG	71 (23.9%)	11 (20.0%)		
Dominant model	GG + GC	157 (52.9%)	34 (61.8%)	0.221	0.693 0.384-1.249
	CC	140 (47.1%)	21 (38.2%)		

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4.10. Association of IL-6 -174G>C polymorphism and HBV

The genetic models between patients and healthy controls. CC+GG genotype showed highest percentage in the HBV positive participants in the over-dominant genetic model even though the results was not statistically significant (p=0.634; OD=0.880 and CI=0.520-1.490). The recessive genetic model was dominated by CC+GC in the HBV positive participants (p=0.296; OD=0.721 and CI=0.390-1.334).

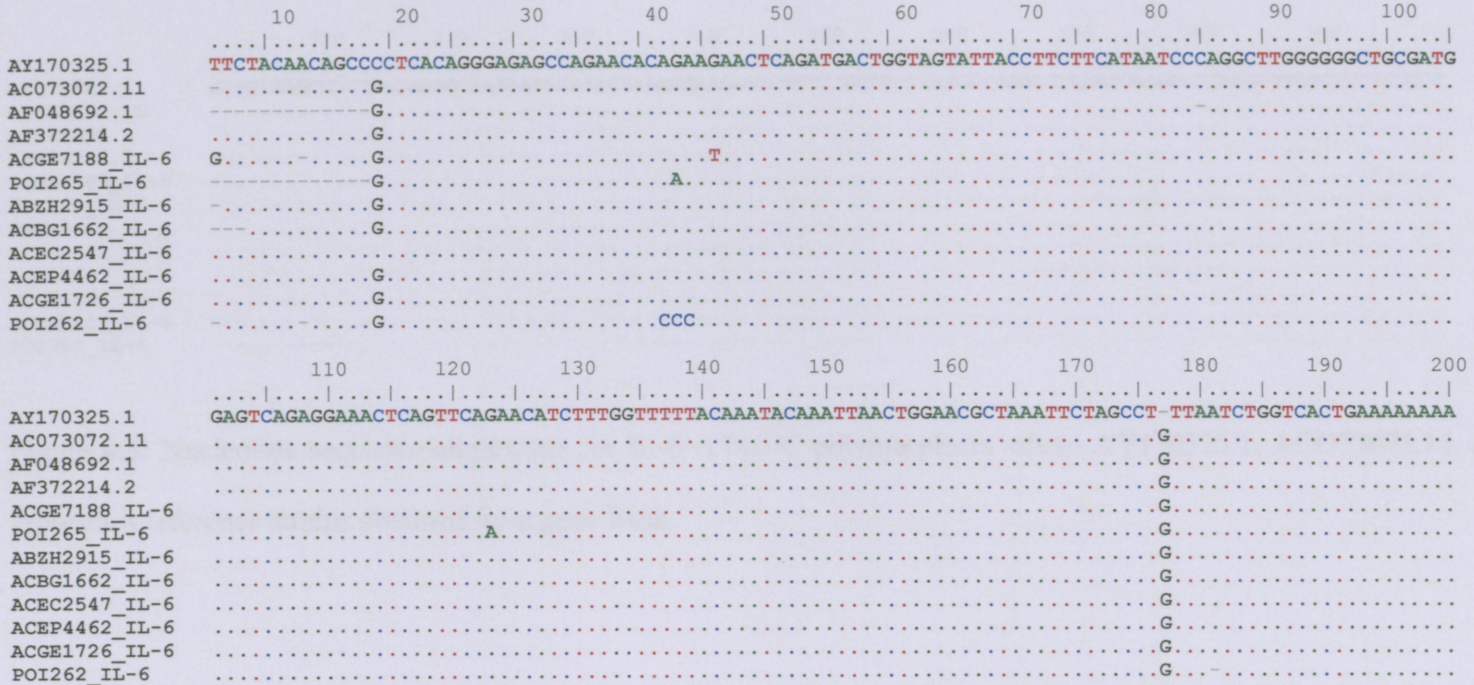
Table 4.8: Association of IL-6 genetic models with HBV

Models		Healthy	HBV	P value	Odd ratio(95% CI)
Over dominant model	GC	77(30.6%)	28(33.3%)	0.634	0.880 0.520-1.490
	CC + GG	175(69.4%)	56(66.7%)		
Recessive model	CC + GC	190(75.4%)	68(81.0%)	0.296	0.721 0.390-1.334
	GG	62(24.6%)	16(19.0%)		
Dominant model	GG + GC	139(55.2%)	44(52.4%)	0.221	1.118 0.682-1.834
	CC	113(44.8%)	40(47.6%)		

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4.11. Sequence analysis

A sequence alignment produced by clustal W multiple aligner for IL-6 -174G/C gene promoter. Referral strain AY170325.1 and sample ACEC2547 shows C SNP at position 19 while others shows G SNP. At position 177 all samples shows G SNP. At position 201 and 202 samples and referral strains shows either AA genotype or TT genotype and AT genotype.



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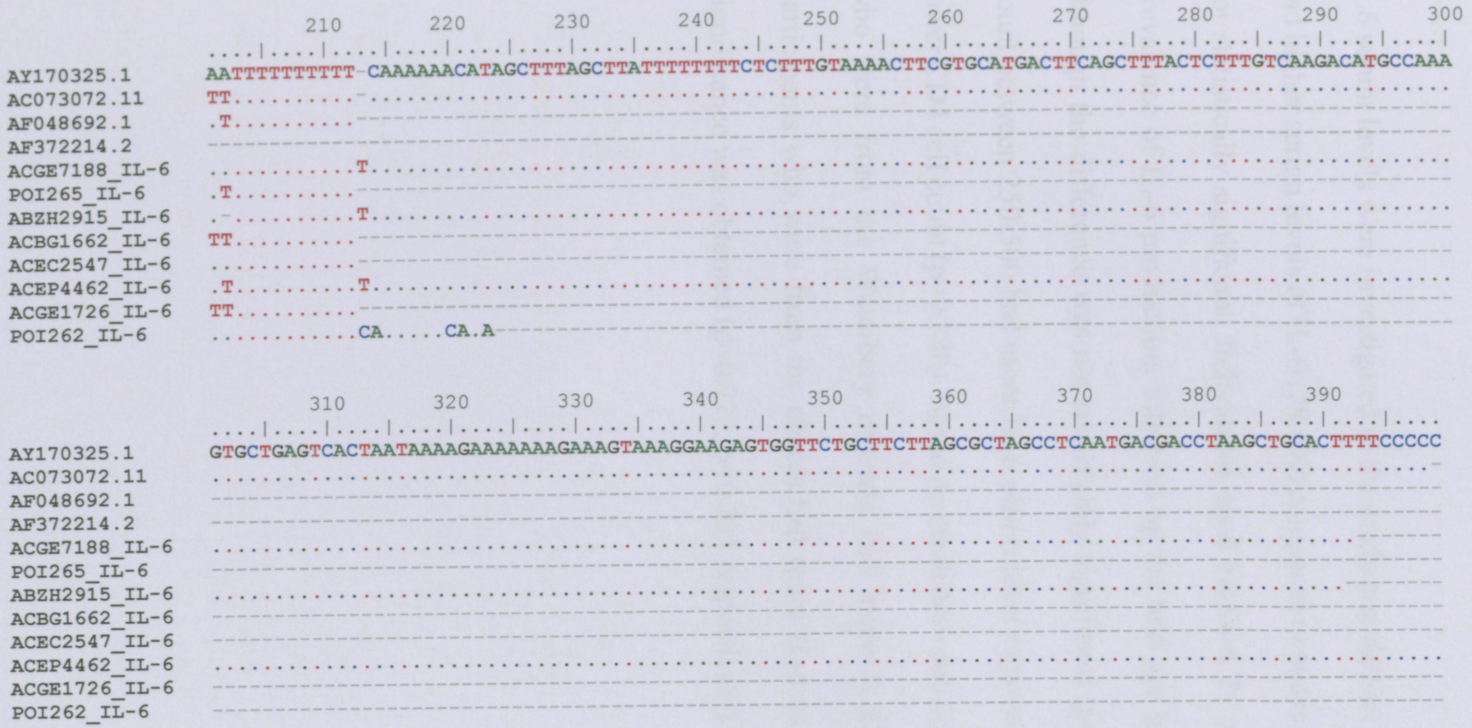


Figure 4.5: Nucleotide sequence alignments for IL-6 -174G/C polymorphism where AY170325.1, ACO73072.11, and AFO48692.1 represents reference strains obtained from gene bank.

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4.12. Distribution of IL-6 levels based on Gender, age, CD4 cell count, district and origin

IL6 serum levels were investigated in the study population. The results showed that more females had higher serum levels of IL-6 (28.4%) compared to males (22.6%). However, the difference was not statistically significant. Individuals aged between 21 and 45 years (27.4%) had the highest prevalence of IL-6 production, followed by the mid age group (0-20) with a 27.0% prevalence although the difference was not statistically significant (p=0.567). Participants with a CD4 cell count between 250-500 had more IL-6 serum level expression (29.9%) compared to those with a lower CD4 cell count (p=0.830). Based on the district and origin of the samples most of the patients who were from the Waterberg districts had more IL-6 serum expression and most of the participants who were from the clinics had more IL-6 expression. In both cases, no statistical significance was obtained (p=0.720, p=0.735 respectively).

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Table 4.9: Distribution of IL-6 levels based on Gender, age, CD4 cell count, district and origin

Characteristics		Low levels of IL-6	High levels of IL-6	Statistics
Gender	Male	82(77.4%)	24(22.6%)	χ^2=1.273 p=0.259
	Female	184(71.6%)	73(28.4%)	
Age group	0-20	27(73.0%)	10(27.0)	χ^2=1.134 p=0.567
	21-45	175(72.6%)	66(27.4%)	
	>46	56(78.9%)	15(21.1%)	
CD4 count	0-250	67(74.4%)	23(25.6%)	χ^2=0.881 p=0.830
	251-500	96(70.1%)	41(29.9%)	
	501-1000	80(74.8%)	27(25.2%)	
	>1001	12(70.6%)	5(29.4%)	
District	Capricorn	132(75.4%)	43(24.6%)	χ^2=0.658 p=0.720
	Sekhukhune	69(75.0%)	23(25.0%)	
	Waterberg	53(74.3%)	88(25.7%)	
Origin	Clinic	166(72.8%)	62(27.2%)	χ^2=0.114 p=0.735
	Hospital	265(73.4%)	96(26.6%	

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4.13. Association of IL-6 levels with hepatitis virus

IL6 serum levels were investigated and associated with the presence or absence of the infection with HCV and HBV. Participants who were positive for HCV had low levels of IL-6 (81.4%) as compared to those who were HCV negative (72.0%), however in all the results no statistical significance was reached. Participants who were positive for HBV had high levels of IL-6 as compared to those who were HBV negative, and the difference was statistically significant (**p=0.001**).

Table 4.10: association of HCV and HBV with IL-6 levels

Characteristics		Low levels of IL-6	High levels of IL-6	Statistics
HCV	Negative	270(72.0%)	105(28.0%)	χ^2=2.691 p=0.101
	Positive	57(81.4%)	13(18.6%)	
HBV	Negative	260(81.3%)	60(18.8%)	**χ^2=51.658** **p=0.001**
	Positive	36(42.4%)	49(57.6%)	

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CHAPTER FIVE
DISCUSSION AND CONCLUSIONS

5.1. Discussion

The natural outcome of HBV and HCV infection varies dramatically among individuals. HCV infection is self-limited in a fortunate minority, while the majority of subjects develop persistent (chronic) infection (Alter et al., 1992). However, infection with HBV in adults progresses to the chronic phase in about 5% (Asselar et al., 2007). Among those individuals with persistent HBV or HCV infection, the majority develop CH, progressive fibrosis and even liver cancer (Bwogi et al., 2009) while, some cases will never progress to any significant liver disease. It remains unknown why the infection frequently turns into CH, and why the outcome with HBV and/or HCV infection varies dramatically (Hoffmann et al., 2001). Cytokines play a crucial role in regulating the immune and inflammatory responses. Polymorphisms in the regulatory regions of the cytokine genes may influence their expression. Therefore, as genetic predictors of the disease susceptibility or clinical outcome, the polymorphisms of cytokine genes are potentially important.

Enough evidence on the prevalence of HCV and HBV in Limpopo Province particularly rural areas, has not been entirely disentangled. Furthermore, the role of host genetic factors that might contribute to the burden of disease with the pathogens has never been studied especially in districts within the Limpopo Province. Therefore there is a need for identification and association of candidate genes that might contribute to the outcomes of the infections with either HCV or HBV.

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The present study aimed to determine the impact of host genetic polymorphisms at IL-6 gene on the occurrence of HCV and HBV.

5.1.1. Seroprevalence of HCV and HBV

Sero-epidemiological studies on HBV and HCV are an important step in formulating preventive strategies. The prevalence rates of these infections vary according to the risk factors involved, socioeconomic status, and initial burden of infectious markers in the community, which vary from one country to another and even between different regions within the same country (Daw et al., 2014). In the present study out of the 455 samples tested 70 were positive for HCV, making an overall sero-prevalence of 15.4%. The results obtained differ substantially from the results that were obtained from other countries for example a study done by Daw et al., (2014) in Tripoli-Libya reported a lower prevalence of 0.9%. Another study in India showed the low prevalence (5.6%) among liver patients (Anbazhagan et al., 2010). These findings suggest the endemicity of HCV infection in the study population. The high prevalence observed in the present study indicates a dire need for HCV screening programs.

It was observed that males (15.1%) were more susceptible to HCV than female (13.2%) among the study population, although the difference was not statistically significant (p=0.629). This concurs with a previous report that male subjects were at a higher risk of developing HBV infection than females (Sud et al., 2001 and Padmapriyadarsini et al., 2006). This could be clarified by the way that the male populace is more inclined to have different sexual accomplices than females (Tremeau-Bravard et al., 2012). In many countries which have HCV screening programs, age is used as a primary selective determinant. In the present study, the highest prevalence was observed

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among participants aged >45, the difference was not statistically significant when compared to other age groups (p=0.076). Nonetheless, Ramarokoto et al., (2008) in their review on the seroprevalence of hepatitis C in urban territories of Madagascar detailed that the predominance did not contrast altogether as indicated by sexual orientation but rather it expanded with age.

In the present study participants with low CD4 cell count had high prevalence of HCV, however the difference was not statistically significant (p=0390). Other studies have reported that people with CD4 cell count above 250 had high prevalence of HCV (Lodenyo et al., 2000; Adewole et al., 2009 and Wondimeneh et al., 2013). This low CD4 cell count in HCV infected may be associated with an increased HCV replication, reflecting the immunosuppressed state. The highest prevalence was observed in Waterberg compared to other districts, however the difference was not statistically significant (p=0.800). The high prevalence of HCV in Waterberg could be identified with financial conditions, as inhabitants of these regions have lower salaries and higher rates of unemployment, and they house huge quantities of youngsters with drugs. Such variety has additionally been accounted for in other Mediterranean nations. In Italy, Greece, France and Spain, the prevalence of the infection differs incredibly starting with one district then onto the next inside a similar nation (Mühlberger et al., 2009 and Daw et al., 2014). We identified a relatively high prevalence of HBsAg among the study population (20.9%). These results are higher than those reported in 2015 for HBV co-infection among HIV-positive women in South Africa (Matthews et al., 2015). The findings from our study are incomparable from other studies done in Lybia and India (Anbazhagan et al., 2010 and Daw et al., 2014). This difference may be due to geographical variation, genetic difference; immunity, other socioeconomic characteristics and sample size variation also may contribute to this difference. However, comparative predominance has been accounted for in South West and Littoral Regions of Cameroon (25.5%), Lagos Nigeria (25.7%),

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Pakistan (24.2%) and Sekota Northern Ethiopia (21.16%) (Magoro et al., 2016; Belo et al., 2000; Khan et al., 2010 and Gebreegziabher et al., 2016).

The seroprevalence of hepatitis B among males and females in our study was 22.0% and 21.1%, respectively. In a study on hospitalized patients in Manipal, Dutta et al., 1993 observed HBsAg positivity of 35.3% in males versus 19.3% in females. Similar findings were also observed in Jaipur, Rajasthan (Sood and Malvankar, 2010). No conceivable clarification has been given for the higher prevalence in men in the all-inclusive community yet likely females clear the HBV all the more productively when contrasted with men (Qamer et al., 2004).

The highest sero-prevalence was observed in age groups >45 years, the difference was not statistically significant when compared to other age groups however (p=0.076). The results from our study are incomparable to a study done in Sekota, Northern Ethiopia by Gebreegziabher et al., 2016 who observed a positivity rate of 14.31 % in the age group between 15 and 45. This most likely reflects ongoing exposure over time, as has been reported by others (Laurent et al., 2010 and Rusine et al., 2013). The highest prevalence was observed in Sekhukhune compared to other districts, however the difference was not statistically significant.

5.1.2. Effects of IL-6 host genetic polymorphism in the study population

These days, knowledge about host control of microbial diseases continue to be gathered. Particular lots of SNPs variants present on genes encoding for immune response effectors are recurrently found associated with a wide range of viral infections (Chinn et al., 2010 and Fumagalli et al., 2010). There is however an intriguing trend to downplay this redundancy and to consider every infection as completely different entity essentially dependent on infectious agent characteristics.

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IL-6 is a circulating cytokine secreted by activated macrophages and lymphocytes, which acts as an important modulator of immune responses (Fabrício-Silva et al., 2015).

In our study population, a higher percentage of CC (35.5%) genotype. These results are completely different when compared to other studies. A study done by Gomes *et al.,* (2014) in Portugal, Porto showed genetic variants of different distribution of GG (37%), GC (54.9%) and CC (7.5%) genotypes in relation with lung cancer. Furthermore, a study done by Gabriela-Teixeira *et al.,* (2014) showed genetic variants of different distribution of GG (76.1%), GC (17.2%) and CC (6.7). This difference may be due to geographical variation and genetic difference. The GC (20.9%) and GG (13.6%) genotypes were overexpressed in participants who were HCV positive as compared to those who were negative but the results did not reach any statistical significance (p=0.189). These results differ with those obtained in a study done by Fabrício-Silva et al (2015) who found a percentage of 43.3% GG, 44.9% GC and 11.8% CC in patients with chronic HCV in Brazil. However, a similar case was observed in the present study where the GG genotypes were overexpressed in patients who were positive for HBV than HCV (45.9% and 6.9%), but the statistical significance was not reached as well in their study (0.684). In the present study there was no association between IL6 -174G/C polymorphism and HBV. However, these results show similarity with those obtained in a study by Park et al., (2003) while investigating IL-6 -174G/C polymorphism on the risk of HBV disease in the Korean population. Our study find no association for IL-6 -174 gene polymorphism under any genetic models with both HCV and HBV.

5.1.3. IL-6 levels in the study population

Clinical research has shown that pro-inflammatory and regulatory cytokines have a prominent role in the pathogenesis of chronic HCV and HBV. Indeed, a positive correlation between pro-

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inflammatory cytokines and liver fibrosis has been reported (Kawakami et al., 2000), whereas modulation of cell-inflicted liver injury by regulatory cytokines leads to milder forms of disease (Napoli et al., 1996 and Gigi et al., 2008). The results showed that more females had higher serum levels of IL-6 (28.4%) compared to males (22.6%). Our data showed that serum levels of IL-6 did not correlate with age or gender. A study done by Behark *et al.,* (2001) showed that IL-6 levels were not significantly different between young and elderly subjects. Another study done by Aricun et al., (2005) showed similar findings.

In the present study, participants who were negative for HCV showed high expression of IL-6 levels. A study done by Teixeira-Carnalho et al., (2014) showed that individuals with HCV had high levels of IL-6. Another study showed that patients with HCV had high levels of IL-6 before and after with interferon-alpha treatment (Gigi et al., 2008). Increased levels of IL-6 have been reported in patients with chronic HBV, cirrhosis and HCC. In the present study, participants who were positive for HBV had high levels of IL-6 as compared to those who were HBV negative, however the results were statistically significant (**p=0.001**). Studies done by Malaguarnera *et al*., 1997 and Song *et al*., 2000 reported that Serum levels of IL6 were found to be significantly increased in patients with chronic hepatitis B. Thus, it is suggested that IL-6 can be a diagnostic marker of chronic HBV infection (Bita Moudi et al., 2016).

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5.2. Conclusions

Infection induced hepatitis is a severe cause of mortality and morbidity especially in developing countries. In this study the seroprevalence of anti-HCV Ab and HBsAg was 15.4% and 21.5 % respectively. These findings suggest the endemicity of HCV and HBV infections. HCV and HBV were both higher in males than in females, this means that male are more prone to have multiple sexual partners and are exposed to drugs. However; the study indicates that hepatitis viral infection is still a public health problem which should be among the prioritized health problems in the country.

Identification of the gene variants that affect susceptibility to diseases is an initial step towards defining the genes and the interactions among their products that determine susceptibility to diseases. This could be potentially be translated into more efficient therapeutic strategies. The distribution of the IL-6 promoter gene at -174G/C polymorphism was different between our population and those described in European studies. This is an important discovery and warrant further investigations. Polymorphism at the IL-6 gene promoter at -174G/C did not show any association with either infections, suggesting that the polymorphism might not have an impact on the occurrence of the infections, however more larger, better designed studies needs to be carried out using appropriate molecular and statistical methods to further analyse the associations. In the present study high levels of IL-6 was not associated with HCV, However, it was associated with HBV. The causal role of IL-6 in HBV can provide valuable information on the pathogenesis of HBV.

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5.3. Recommendations

Hepatitis viral infection has also shown a higher prevalence among males, which calls for an urgent need for males to be screened for STI's and campaigns for males to use condoms if they have multiple partners.

Hepatitis viral infection screening programs should also be prioritized following possibility of lack of information concerning the infection and its devastating long term consequences.

Vaccination should also be prioritized so that the community can be protected against this debilitating burden of disease, therefore candidate molecules that can be used as vaccine targets should be urgently identified studied and tested.

Genetic association studies usually requires a larger set of samples, we suggest that additional genetic studies involving multiple-locus analyses should be considered to improve our understanding of the interaction between pro-inflammatory cytokine polymorphisms that impact Hepatitis viral infection progression and to provide scientific support for the establishment of novel immunomodulatory treatments.

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5.4. Limitation of the study

Epidemiology requires a larger set of samples, so one of the limitations in the present study is the sample size which was smaller and could not be used as a representative of the whole population in the district covered in the study population.

Characterization of genetic profiles and analysis of differences between ethnic groups should provide a better understanding of their disparities and genetic susceptibility to disease. In the present study the data about the ethnic group was missing.

Lack of access to clinical data was also a problem in the present study. However during analysis this was considered.



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CHAPTER SIX

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