PREVALENCE AND MOLECULAR CHARACTERIZATION OF HEPATITIS C VIRUS (HCV) AMONG PATIENTS IN RWANDA

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PAN AFRICAN UNIVERSITY INSTITUTE FOR BASIC SCIENCES, TECHNOLOGY AND INNOVATION

2017
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MB 300-0015/2015

A thesis submitted to Pan African University, Institute for Basic Sciences Technology and Innovation in partial fulfillment of the requirement for the degree of Master of Science in Molecular Biology and Biotechnology

2017
DECLARATION

This research thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

I dedicate this research work to my husband, Dusabe Sosthene and my lovely son, Avi Dillon Dusabe. Your encouragement, patience and constant support have brought me this far.
ACKNOWLEDGMENTS

First and above all I thank almighty God who strengthened me throughout this research. I would like to express my gratitude to my supervisors Dr Fabien Ntaganda and Professor Naomi Maina for their help, useful comments, remarks, motivation, enthusiasm, and immense knowledge. Their guidance helped me in all the time of research and writing of this thesis. I would like to thank the member of the reading committee, Dr. John Kagira for his excellent advise and detailed review during the preparation of my paper. I greatly appreciate the excellent assistance, advice, support of Dr Ben Karenzi and Dr Emmanuel Ndahiro from Rwanda military Hospital during my thesis preparation. I thank E.M.Talyor, for his advice and his friendly assistance with various problems all the time, especially for his help with the paperwork.

Furthermore I thank the classmates for the help and assistance. I thank Mr Boniface Mathendu from Biozeq molecular lab and Rwanda Military hospital staff for your excellent technical assistance in the Lab. I also appreciate the financial support of JICA Project Office, JKUAT and the Pan African University Scholarship Programme which helped me to carry out my Msc study and research.

Last but not the least; I would like to thank my husband and my family for continuous wise counsel. They have been there for me.
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LIST OF ABBREVIATION

ALT - Alanine aminotransferase

cDNA - complementary DNA

kCHC – Chronic Hepatitis C

CLDN-1 - claudin-1

DAA - Direct Acting Antiviral

ECDC - European Centre for Disease Prevention and Control

EIA - Enzyme Immunoassay

HCC - Hepatocellular Carcinoma

HCV – Hepatitis C Virus

HVR - Hypervariable Region

IDU - Intravenous Drug Use

IFN – Interferon

IgG – Immunoglobulin G

IRES - Internal Ribosomal Entry Site

LDLR - Low Density Lipoprotein Receptors

NAT - Nucleic Acid Test

NNPI - Non-nucleoside Polymerase Inhibitor

NPC1L1 - Niemann-Pick C1-Like 1

NPI - Nucleoside Polymerase Inhibitor
NS – Nonstructural protein

OCLN - occluding

ORF – Open Reading Frame

PCR – Polymerase Chain Reaction

RBC - Rwanda Biomedical Center

RMH-Rwanda Military Hospital

RDT - Rapid Diagnostic Test

RIBA - Recombinant Immunoblot

RMH – Rwandan Military Hospital

RNA – Ribonucleic Acid

RTK - Receptor Tyrosine Kinases

RT-RNA PCR – Reverse-Transcription RNA PCR

SRB1 - Scavenger Receptor Class B type I

SVR - Sustained Virologic Response

UTR - Untranslated Region

WHO – World Health Organization

NISR – National Institute of Statistics of Rwanda

FBC – Full Blood Count

ESR – Erythrocyte Sedimentation Rate
ABSTRACT

Hepatitis C virus causes a serious liver infection and affects approximately 3% of the world's population. In Rwanda, the prevalence of the disease is not well understood. The study was aimed at investigating the active prevalence of hepatitis C virus, characterize genotypes circulating in the general population and analyze haematological changes among hepatitis C virus infected persons in Rwanda. A total of 324 patients attending Rwanda Military hospital were randomly selected. A structured questionnaire was administered to each consenting participant to determine the risk factors. Blood was collected, screened for anti-HCV antibodies and seropositive samples subjected to polymerase chain reaction method to confirm active infection. Hematology abnormalities in the HCV infected patients were also investigated. Anti-HCV antibody was found in 16.0% of the participants and 9.6% of the total participants had active HCV RNA infection. Prevalence was highest (28.4%; 19/67) among participants above 55 years and least (2.4%; 3/123) among younger participants (18-35 years). There was a significant relationship (p = 0.031) between place of residence and HCV infection with residents of Southern Province having significantly higher prevalence (13.1%; 13/99). The hematological abnormalities observed in the HCV infected patients included; leukopenia (48.4%; 15/52) neutropenia (6.5%; 2/52) and thrombocytopenia (25.8%; 8/52). There is a significant association of HCV infection and thrombocytopenia (P=0.033). The study showed a high rate of HCV infection in Rwanda and was significantly higher in the older population (> 55 years) and exposure to injection from traditional Doctors was identified as a significant (p=0.036) risk factor of infection.
Further studies to determine the factors causing the high prevalence with an aim of instituting intervention strategies are recommended.
ABSTRAIT

Le virus de l’hépatite C (HCV) provoque une grave infection de foie. Environ 3% de la population mondiale est infectée par l’HCV. Au Rwanda, le nombre de population infectée par l’HCV est inconnu. L’objectif de cette étude est d’enquêter sur le nombre de la population infectée par hépatite C, caractériser les génotypes circulant parmi la population en général et analyser les changements hématologiques chez les personnes infectées par l’hépatite C au Rwanda. Un nombre total de 324 patients visitant l’hôpital militaire du Rwanda a été sélectionné au hasard. Un questionnaire a été utilisé pour déterminer les facteurs qui contribuent à l’infection de chaque patient. Le sang a été prélevé, testé pour les anti-HCV anti-corps et les échantillons séropositifs sont soumis à l’amplification en chain par la polymérase (ACP) pour confirmer une infection active. Des anomalies hématologiques chez les patients infectés par HCV sont également étudiées. Anti-HCV anti-corps a été trouvé chez 16.0% des participants et 9.6% du total des participants avaient une infection active. La prévalence de l’hépatite C était la plus élevée (28.4%; 19/67) chez les participants de plus de 55 ans et elle était moins élevée (2.4%; 3/123) chez les jeunes participants (18-35years).

Il y avait une relation significative (p = 0.031) entre le lieu de résidence et l’infection HCV des résidents venant du Sud du pays ayant une prévalence la plus élevée (13.1%;13/99). Des anomalies hématologiques qui ont été observées chez les patients infectés par HCV inclus: leucopénie (48.4%; 15/52), neutropénie (6.5%; 2/52) et thrombocytopenie (25.8%; 8/52). Il y a une relation significative entre l’infection HCV et thrombocytopenie (P=0.033). L’étude a montré que l’infection HCV au Rwanda est
plus élevée et surtout la population plus âgée (> 55 years) est la plus exposée et ainsi que l’exposition à l’injection par des docteurs traditionnels a été identifiée comme un facteur de risque important de l’infection (p=0.036). D’autres études pour déterminer les facteurs provoquant la prévalence ayant pour objectif d’instituer des strategies d’intervention sont recommandées.
CHAPTER ONE

1.0. INTRODUCTION

1.1. Background Information

Hepatitis C, a serious viral disease of the liver and caused by hepatitis C virus (HCV) infection is a major health issue of global concern (Mohamed et al., 2015; Stanaway et al., 2016). Recent estimates suggest that about 130-150 million of people across the globe are living with chronic HCV infection (WHO, 2015) and the annual mortality due to HCV related liver diseases is about 350,000–500,000 (Graham & Swan, 2015; Lozano et al., 2012). Infection with HCV causes acute infection that is usually asymptomatic (Blackard, Shata, Shire, & Sherman, 2008) and chronic hepatitis C (CHC) infection (Rosen, 2011). Recent studies have shown that CHC infections are most predominant in patients older than 60 years (Davis, Alter, El-Serag, Poynard, & Jennings, 2010). In contrast, host factors such symptoms of illness, being female, younger age and strong adaptive immunity and other genetic factors (ILB28) among persons with acute infections have been shown to favour spontaneous clearance of the virus before progression into chronic infection (Chung, 2005; Leone & Rizzetto, 2005). In the absence of these protective factors untreated acute infections result in chronic infections and patients with CHC infections gradually develop liver fibrosis which can lead to cirrhosis, liver decay and hepatocellular carcinoma (HCC) (Hajarizadeh, Grebely, & Dore, 2013).
The global distribution and prevalence of HCV infections and genotypes vary considerably among geographical regions (Gower, Estes, Blach, Razavi-Shearer, & Razavi, 2014; Messina et al., 2015). The infecting HCV genotypes influence the appropriate HCV treatment medication. There are six major HCV genotypes that have been identified (Messina et al., 2015; Zein & Persing, 1996). However, available evidence indicates that, as many as 11 genotypes may exists (Franciscus, 2014; Simmonds, 1999) and each genotype has multiple subtypes (designated a, b, c etc.). In addition, there are about 100 different strains (Ohno et al., 1997). A recent study shows that, HCV genotypes 1 and 3 are the two most prevalent genotypes globally and account for about 76.3% cases of HCV in the world (Messina et al., 2015). However, most cases of HCV caused by these two genotypes are found in East and South Asia respectively. East Asia is also burdened with genotypes 2 and 6 and the two collectively account for about 14.5% cases of global HCV infections. The global cases of HCV due to genotypes 4 and 5 are approximately 8.3% and less than 1% respectively. Africa and Middle East have the most cases of genotypes 4 and 5 (Messina et al., 2015). However, genotype 5 is considered as the least prevalent globally but most endemic in sub-Saharan Africa regions (Messina et al., 2015).

Globally, the prevalence of HCV infection is highest in Egypt (Karoney & Siika, 2013; Lavanchy, 2011; WHO, 2015). According to a recent review publication, the HCV prevalence in Egypt is estimated at 17.5% which is believed to be due to iatrogenic transmission from historical use of contaminated needles (Mohamoud et al., 2013). In Africa context, Egypt is followed by Cameroon with a prevalence of 13.8%, and
Burundi with a prevalence of 11.3%. However, countries with the least prevalence of less than 1% include Zambia, Kenya, Malawi and South Africa (Karoney & Siika, 2013).

In Rwanda, the prevalence of HCV infection among the general population is unknown. However, HCV infection prevalence has been reported for specific groups of the populations such as healthcare workers (1.3%) and HIV-1 positive pregnant women (4.9%) (Kateera et al., 2015; Pirillo et al., 2007). Although an estimate of 4.9% has generally been reported (Lavanchy, 2011) but a recent review indicates this figure could be an underestimation (Karoney & Siika, 2013). This is could be caused by challenges faced in providing HCV care especially in many developing countries. Challenges such as high cost of screening and related medical care plus inadequate knowledge and awareness of hepatitis C among healthcare providers result in under-diagnosis as well as under-reporting of infections (Averhoff, Glass, & Holtzman, 2012).

Unlike the developed countries, where the mode of HCV transmission is mainly through intravenous drug use (IDU) (Aceijas & Rhodes, 2007; Williams, Bell, Kuhnert, & Alter, 2011), in most developing countries contraction of HCV occurs through exposure to infected blood and blood products at various healthcare facilities and localities. Such contamination usually occurs through unsafe injection, blood transfusions, organ transplants and sometimes through mother to child transmission or the sharing needles among IDUs (Hauri, Armstrong, & Hutin, 2004; Prati, 2006). Though sexual contacts may pose some degree of threat to acquiring infection. This implies that, contracting HCV infection is not considered as a high risk. Also, the risk
increases in case of heterosexual contact (Stulhofer, Graham, Bozicević, Kufrin, & Ajduković, 2007).

The screening and diagnosis of HCV infection can be done during both the acute and chronic phases. However, since majority of HCV infections are usually asymptomatic (Leone & Rizzetto, 2005; Okanoue et al., 2005), they are rarely detected during the acute phase. This is often the same case even people who go on to develop chronic HCV infection because the infection remains asymptomatic for decades until symptoms develop and additional complications are observed including serious liver damage (Okanoue et al., 2005; WHO, 2015).

The diagnosis of hepatitis C is based on the detection of both anti-HCV antibodies and HCV RNA in the presence of biological or histological signs of chronic hepatitis (Daw, 2014). Various serological assays that detect anti-HCV IgG in sera have been developed and improved over the years. These assays include immunoassays such as third generation enzyme immunoassay (EIA), immunoblot assays like recombinant immunoblot assay-3 (RIBA-3), and the most recent immunochromatography-based rapid tests (Abdel-Hamid et al., 2002). When Anti-HCV antibodies are detected, a molecular-based testing for HCV RNA is subsequently carried out to confirm the presence of active HCV infection (Bajpai, Gupta, & Choudhary, 2014). Where anti-HCV antibody is positive but a confirmation with molecular-based method is negative, this suggests a past (inactive) infection. In such cases patients are retested after 3 months, since HCV antibodies can be detected months after patient’s body has cleared the viruses. This phenomenon usually occurs in persons with strong immune-response or protective
genetic factors. Similarly, due to the window period between HCV infection and antibody detection, a negative anti-HCV test when confirmed with molecular-based method may be positive and indicate an early infection where antibody levels are below the detection threshold (Seremba et al., 2010).

Chronic infections with HCV are usually marked with hematological abnormalities such as neutropenia, anemia, and thrombocytopenia (Kedia et al., 2014). These abnormalities are commonly found in patients undergoing anti-retroviral treatment (Kedia et al., 2014; Tawadrous, Aziz, Amin, Eldemery, & Mostafa, 2012). Other studies have shown that low platelet count, low hemoglobin and high levels of creatinine are some predicting factors associated with the development of hematological abnormalities in HCV patients (Lashin et al., 2013; Nachnani, Rao, Bulchandani, Pandya, & Alba, 2010).

Rwanda is facing an increasing problem of HCV infection among the population and the number of HCV infected people who are receiving treatment is low. This is due to the high cost associated with drug treatment. To circumvent this challenge, the government has put in measures to improve disease control and create awareness among the people. Some of these measures include testing the disease at the National Blood Transfusion Center, Kanombe Military Hospital and other governments and private Hospitals. The ministry of health is curbing on the use of unsterilized materials such as needles and visiting traditional practitioners. As a result some existing health facilities in the country have shown promise in increasing access to HCV treatment (Kirk CM & Bucher HC, 2015).
There is currently no vaccine available for hepatitis C. However, there are many drugs available for treatment. Treatments of HCV are based on interferon and four classes of direct acting antivirals (DAAs). The four classes of DAAs are HCV; (a) protease inhibitors targeting NS3/5 protein, (b) nucleoside polymerase inhibitors (NPIs) targeting NS5B proteins, (c) non-nucleoside polymerase inhibitors (NNPIs) targeting NS5B protein, and (d) NS5A inhibitors (Kiser & Flexner, 2013; Poordad & Dieterich, 2012). But treatment with combination therapy such as pegylated interferon in combination with ribavirin has been shown to offer better treatment responses than monotherapy (CDC, 1998). Thus, the ultimate goal of HCV treatment is to achieve significant sustained virologic response rate (SVR) by interfering with HCV replication (Lindsay, 2002).

1.2. Statement of the Problem

The severity of hepatitis C disease can range from a mild illness to a serious condition that can lead to chronic liver disease, end-stage cirrhosis, and liver cancer (Kretzer et al., 2014). In East Africa, the HCV genotype 4 is the most prevalent and account for most cases of infections with Burundi leading with an estimated 11.3% seroprevalence (Petruziello, Marigiano, Loquercio, Cozzolina, & Cacciapuoti, 2016). Rwanda with prevalence between 4.1% and 4.9% having been reported in various review publications and other countries such as Kenya has less than 1% prevalence (Karoney & Siika, 2013; Lavanchy, 2011; Madhava, Burgess, & Drucker, 2002).

The prevalence in Rwanda's general population is unknown (Kirk CM & Bucher HC, 2015). Few studies conducted so far focusing on only specific groups of the populations
such as pregnant women, tuberculosis patients and HIV infected patients (Kateera et al., 2015; Kirk CM & Bucher HC, 2015; Pirillo et al., 2007; Rusine et al., 2013). This scarcity of information shows how the disease has been neglected in Rwanda. It is believed that, prevalence of HCV infection reported in various studies is even an underestimation (Karoney & Siika, 2013). This is because, most people especially those in rural areas have no access to health care facilities for routine health checkup. Moreover, screening and testing of HCV is not free in Rwanda and therefore the cost associated with early detection of infection alone deters many from going for voluntary testing to know their status.

Hematological abnormalities such neutropenia, leukopenia, anemia and thrombocytopenia have been reported among HCV patients undergoing treatment as well as pre-treatment patients (Dieterich & Spivak, 2003; Streiff, Mehta, & Thomas, 2002). However, this phenomenon among HCV infected patients in Rwanda is yet to be investigated. A few studies have been done to determine the genotypes circulating in the Rwandese population but none of them have characterized the subtypes of those genotypes. Both HCV genotypes 1 and 4 have been identified in Rwanda (Kamal & Nasser, 2008). However, the various subtypes of these genotypes specific to the population have not been identified.
1.3. Justification of the study

Although many review publications on global epidemiology of HCV put HCV prevalence estimate in Rwanda at between 4.1% and 4.9% (Karoney & Siika, 2013; Lavanchy, 2011), data on HCV infection specifically in Rwanda is scarce. Besides, the few studies on HCV prevalence in Rwanda are based on anti-HCV antibody testing with special categories of participants. This study combined both anti-HCV test and HCV RNA PCR test to estimate the prevalence of HCV infection in Rwanda. The HCV RNA PCR testing was used as a confirmatory test because, many studies have shown that HCV antibody testing under African setting is flawed with many false results leading to wrong estimation of HCV prevalence rate. The findings could help the Rwanda decide on the introduction of HCV RNA PCR testing in the country and contribute to increase HCV. Questionnaires introduced during the study will help establish how HCV infection, genotypes and subtypes vary with demographic, epidemiologic, and other clinical variables in the study area.

Phylogenetic analysis of the nucleotide sequences, genotypes and subtypes of hepatitis C virus (HCV) identified and characterized will provide useful baseline that may contribute to the development of an effective vaccine against infection with HCV. Moreover, differences among HCV genotypes discovered in this study will provide investigators as an epidemiologic marker that may be used to trace the source of HCV infection in the country and also help update the existing data on the geographical distribution of HCV genotypes. In this study, the hematological profile was assessed in HCV patients to aid in early diagnosis of HCV infection and monitoring of prognosis.
1.4. Objective of the Study

1.4.1. General objective

To determine the prevalence and characterize HCV infections among Patients as well as assess the haematological profile of HCV Infected Patients in Rwanda

1.4.2. Specific objectives

i. To determine the seroprevalence and active HCV prevalence in patients attending a hospital in Rwanda.

ii. To assess the hematological profile of HCV positive samples.

iii. To characterize the HCV genotypes and subtypes circulating in the population.
CHAPTER TWO

2.0. LITERATURE REVIEW

2.1. Hepatitis C Virus (HCV)

2.1.1. The Structure of HCV

The HCV is an enveloped particle of virus belonging to the Flaviviridae family, genus Hepacivirus and causes acute and chronic liver disease in humans. Like all other members of the family flaviviridae, HCV is a positive stranded RNA genome virus with the RNA genome contained in a nucleocapsid made up of core proteins enveloped in a lipid bilayer (Lindenbach & Rice, 2005). The RNA genome ranges in size from 9.6 to 12.3 thousand nucleotides (nt), with an open reading frame (ORF) encoding a polyprotein of 3000 amino acids (aa) or more. The ORF is flanked in 5’ and 3’ by highly conserved untranslated regions (UTR) of 95–555 and 114–624 nt in length, respectively and play an important role in polyprotein translation and RNA replication (Chevaliez & Pawlotsky, 2006). Additionally 5’ UTR contains an internal ribosomal entry site (IRES) to direct translation of polyprotein into at least 10 structural proteins (Dubuisson, 2007). The ORF encodes both the structural proteins and nonstructural proteins (Choo et al., 1991; Miller & Purcell, 1990; Thurner, Witwer, Hofacker, & Stadler, 2004). Depending on the genotype, HCV ORF contains 9024 to 9111 nt and encodes 3 structural proteins (S) namely C or core, E1 and E2 which act as entry factor and 6 nonstructural (NS) proteins, NS2, NS3, NS4A, NS4B, NS5A and NS5B responsible for virus replication and viral life cycle. Another seventh protein, p7, also appears to have a
non structural function. It may be a member of the viroporin protein family and play a role as a calcium ion channel (Figure 2.1) (Abdel-Hakeem & Shoukry, 2014; Chevaliez & Pawlotsky, 2006; Gonzalez & Carrasco, 2003).

**Figure 2.1:** The hepatitis C virus genome.

Source: Protective immunity against hepatitis C many shades of gray (http://dx.doi.org/10.3389/fimmu.2014.00274).

Flanking the ORF are the 5’UTR and the 3’UTR with IRES to direct translation of structural proteins; core, E1, and E2. The structural proteins are cleaved at cleavage sites (indicated by violet arrows) by peptidases into independent proteins. The ORF also codes for nonstructural proteins; p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. The
polyproteins have various cleavage sites (indicated by grey and purple arrows) at which cleavages into individual proteins occur. NS2 has an autoprotease activity.

2.1.1. The Life Cycle of HCV

The HCV life cycle begins with viral attachment, entry and fusion. This is followed by viral translation and replication and ends with viral assembly and release. HCV displays liver tropism and therefore the life cycle begins with viral entry into the host hepatocytes. The HCV particles in circulation cross the fenestrated endothelium of the liver sinusoids and come into contact with the hepatocytes. Upon coming into contact, the viral particles interact through initial attachment factors and receptors on the surface of the liver cells (Dubuisson & Cosset, 2014). This attachment onto hepatocytes was initially thought to involve the hypervariable region (HVR) in HCV E2 glycoprotein (Flint & McKeating, 2000) facilitated by the heparan sulfate proteoglycans expressed on hepatocyte surface (Lefèvre, Felmlee, Parnot, Baumert, & Schuster, 2014; Shi, Jiang, & Luo, 2013). However, current work by Shi et al., (2013) suggest that ApoE, rather than HCV E2 glycoproteins could be involved in this initial contact. Also, low density lipoprotein receptors (LDLR) have also been proposed to promote HCV cellular entry. The basis for this proposal is on the fact that, HCV particles interact with lipoproteins. Studies have shown that HCV-LDLR interaction may be non-productive to viral entry as it may result in viral particle degradation (Agnello, Abel, Elfahal, Knight, & Zhang, 1999).
Following attachment to the cell surface, the HCV particle interacts with series of specific cellular receptors and entry factors through a multistep process. Among these entry factors identified, those essential for HCV include; the scavenger receptor class B type I (SRB1), CD81, tight junction proteins, claudin-1 (CLDN1) and occludin (OCLN) (Dubuisson & Cosset, 2014). Others include, the receptor tyrosine kinases (RTK) epidermal growth factor receptor (EGFR), ephrin receptor A2 (EphA2) and Niemann-Pick C1-like 1 cholesterol absorption receptor (NPC1L1) (Dubuisson & Cosset, 2014; Kim & Chang, 2013). After binding to several components of the host cell, HCV particle is internalized by clathrin-mediated endocytosis and fusion takes place in early endosomes (Blanchard et al., 2006).

The entry of HCV is followed by RNA translation and replication. Synthesis of new viral RNA occurs in a highly structured replication complex that consists of NS3, NS4A, NS4B, NS5A, and NS5B. The viral RNA is translated by host machinery into a polyprotein, which is cleaved during and after translation by both host and viral-encoded proteases into 10 mature viral proteins, including a number of nonstructural (NS) proteins. One of the viral proteases involved in this post-translational processing is a heterodimeric complex of the NS3 and NS4A proteins (NS3/NS4A). NS3 possesses the proteolytic activity and NS4 is a membrane protein that acts as a cofactor. NS5B is an RNA-dependent RNA polymerase that is essential for viral replication. NS5A has a presumptive role in the organization of the replication complex and in regulating replication. It is also involved in assembly of the viral particle that is released from the host cell (Dubuisson & Cosset, 2014). The last stage of the viral cycle is the assembly
and release of viral particles. Though this process of HCV assemble and release is not well understood, it is believed that the process is closely associated with lipid metabolism and that interaction of HCV core protein with low densities (LDs) is essential for recruiting other viral components involved in virion assembly (Miyanari et al., 2007). Apart from the core protein, the envelope glycoprotein complex is also a major component of the viral particle. During viral assembly, the HCV E1/E2 glycoprotein complex must migrate to the assembly site which is the endoplasmic reticulum (ER). Studies have shown that non-structural proteins NS2 and p7 interacts with E1/E2 to aid E1/E2 heterodimer migration at the virion assembly site (Jirasko et al., 2010). Another nonstructural protein that is involved in viral assembly is NS5A. The C-terminal domain of NS5A protein interacts with the LD-bound core protein and this is a key step in HCV assembly. Many other nonstructural proteins have also been implicated in the viral assembly process. The assembly of HCV is followed by budding in the ER. After assembly and budding, the HCV particles are then released via the secretory pathway (Coller et al., 2012) as shown in figure 2.2.
2.1.2. The Genome and Variability of HCV

The analysis of the hepatitis C genome is done by considering different factors: intergenotypic - finding in genomic regions that are conserved within the same subtype but are distinct between subtypes, intragenotypic- the regions of viral variability within the same genotype or subtype and intrahost- variability is where viral genomic variability occurs within the same viral subtype and also the same host in individual (Humphreys et al., 2009). The hepatitis C genome was first determined by Choo and his
colleagues in 1991 (Choo et al., 1991). After determination of HCV genomes, other studies were carried out by isolating and sequencing the HCV from the different part of the world. Substantial variability has been found throughout the genome. This has been observed at E1, the NS3 and NS5B regions and N-terminal part of the E2 and NS1 region is a hypervariable region (HVR). The 5' NC and NS4B regions are the most conserved as there are no reliable sequence polymorphisms in these regions. This observed variation is due to the fact that, HCV RNA polymerase lacks a proof reading ability during viral replication and thus resulting in high level of transcription errors made by the viral polymerase (Pozzetto, Bourlet, Grattard, & Bonnevial, 1996).

2.2. The HCV Genotypes and Global Distribution

Knowledge about HCV genotype which is defined as the different genetic variations or strains of HCV play significant role in the treatment of HCV infection (Franciscus, 2014). The genotype has consequences on treatment duration, dose of treatment and the kind of combination needed to achieve high SVR (Franciscus, 2014). Currently six major hepatitis C genotypes (designated 1-6), each comprising multiple subtypes (designated a, b, c, d etc.) have been identified worldwide (Messina et al., 2015; Zein & Persing, 1996). However, the genotypes with worldwide distribution are genotypes 1, 2 and 3. These genotypes respectively account for approximately 83 million, 17 million and 54 million cases of HCV infection globally (Messina et al., 2015). Genotype 1 with the two subtypes; 1a and 1b and it is mostly found in East Asia where more than one-third of HCV cases due to genotype 1 are reported. The subtypes 1a and 1b are also the most predominant genotypes in the United States with subtype 1a as the most common
(Franciscus, 2014; Zein et al., 1996). With 17 million cases globally, genotype 2 with subtypes 2a, 2b and 2c is also mainly contributed by East Asia. According to a review paper by Gower et al., (2014) subtypes 2a and 2b are also common in North America, Europe and Japan, while genotype 2c is found in Northern Italy. Genotype 3, the second most common genotype globally is most endemic to South Asia with this region alone accounting for about three-quarters of the global burden of the disease associated with genotype 3 infections. Genotypes 4, 5 and 6 each account for an estimated 15 million, 1 million and 9.8 million global HCV cases respectively. The North Africa and Middle East account for the largest number of HCV cases.

However, it is worth noting that, the distribution of these genotypes globally is affected by the existence of significant regional, country and local variations. For instance, the study by Gower et al. (2014) showed that, infections in North America, Latin America, and Europe were predominately G1 (62–71%) with G1b accounting for 26%, 39%, and 50% of all cases respectively. North Africa and the Middle East had a large G4 population (71%), which was attributable to the high prevalence of G4 in Egypt. Exclusion of Egypt resulted in genotype 4 accounting for 34% of all infection and the genotype distribution of this region was dominated by G1 (46%). Asia was predominately G3 (39%) followed by G1 (36%), largely driven by the HCV infections in India and Pakistan. G1b accounted for 25% of all infections in this region. In Australasia, G1 dominated (53%), followed by G3 (39%) (Figure 2.3).
In Africa, a recent review study by (Hanafiah, Groeger, Flaxman, & Wiersma, 2013) shows that, all the major genotypes except genotype 6 are present on the continent but vary in their distribution across the five regions. They also showed that, whereas genotypes 1 and 4 are found in all the five regions, genotype 5 is found only in two regions; Southern and Eastern sub-Saharan Africa. In Central sub-Saharan Africa, the majority of cases of HCV are caused by genotype 4 whereas genotype 5 account for over two-thirds HCV cases in Southern sub-Saharan Africa (Messina et al., 2015). Western sub-Sahara and Eastern sub-Sahara Africa regions are predominantly burden by genotypes 2 and 1 infections respectively. However, the majority of cases due to genotype 1 and 2 infections in found in the Northern Africa. With the few study on distribution of HCV genotypes conducted in Eastern sub-Saharan Africa, Rwanda has

**Figure 2.3:** Global distribution of HCV genotypes and subtypes

genotype 4 as the most prevalent genotype, Ethiopia has genotypes 1 and 2, Mozambique genotype 1 and Madagascar has genotypes 1 and 2 (Gower et al., 2014; Iles et al., 2014)

2.3. Hepatitis C Epidemiology

Hepatitis virus infection continues to be a problem in most countries of the world. There is a large degree of geographic variability in its distribution (Khageshan, Kulkarni, & Prabhu, 2015). The progression of HCV infection is still high. According to WHO, around 3 to 4 million people are infected annually (Shepard, Finelli, & Alter, 2005). Many countries are at high risk and the rate of mortality is high. An estimate of 3% representing 150-200 million of people are HCV chronic infected (Hanafiah et al., 2013). The HCV infection resulting in chronic infection is usually serious and leads to death. For instance in 2010, it was reported that 16000 people died with acute infection while 196,000 deaths occurred from HCV chronic infection (Lozano et al., 2012). This infection is very high in Africa and Asia. In Africa, 5.3% people are infected with the greatest prevalence occurring in Central Africa and West Africa and the lower prevalence estimate found in Southern and East Africa (Shepard et al., 2005).

In Central and East Asia greater than 3, 5% of people are infected (World Health Organisation, 2002). In American, countries such as North American, Central and Southern Latin America the number of HCV infected people is low as it is less than 1.5% but United State show a bit high rate. According to European Centre for Disease Prevention and Control (ECDC) in the European region, approximately 9 million people are chronically infected with HCV (Mühlberger et al., 2009) and according to the review
report by (Cornberg et al., 2011), the prevalence of HCV in several countries and the lowest HCV prevalence estimates were from northern European countries, whilst the highest prevalence estimates were from Romania and rural areas in Greece and Italy, as well as portions of Russia. Globally, the countries with the highest prevalence of HCV infection disease are Egypt 17.5%. Pakistan (4.8%) and China (3.2%) (WHO, 2011).

2.4. The Mode of Transmission of HCV Infection

Hepatitis C infection is a liver disease and causes the inflammation of liver. The HCV can lead either to acute or chronic illness. In acute hepatitis, the presence of clinical signs or symptoms occurs for a period of 6 months or lesser after initial contact with the virus (Blackard et al., 2008). However, studies have shown that during acute infections, the disease is usually asymptomatic and infection rarely diagnosed (Chung, 2005). Acute infection however, becomes a problem and risky when the HCV infection is not resolved within 6 months as there is higher chance of resulting in chronic HCV infection. Thus, chronic hepatitis is defined as hepatitis that lasts for more than a period of six months and can eventually lead to cirrhosis and hepatocellular carcinoma. About 15%–45% of patients with acute infection are usually cured spontaneously without taking treatment while the remaining 55–85% infected people develop chronic HCV infection which can result in severe liver complicated such cirrhosis and liver cancer (WHO, 2015). Moreover, Hepatitis C may be associated with autoimmune diseases such as Sjögren’s syndrome and sialadenitis, idiopathic pulmonary fibrosis, polyarteritis nodosa, porphyria cutanea tarda, and a variant of autoimmune hepatitis associated with the presence of anti-kidney and liver microsomal autoantibodies (Khattab, Eslam, & Alavian, 2010).
Transmission occurs through direct contact with an infected person’s blood or certain body fluid such as semen. Blood transfusion, injecting-drug use, employment in patient care or clinical laboratory work, exposure to a sex partner or household member who has had a history of hepatitis, exposure to multiple sex partners, and low socio-economic level are risk factors associated with infection (CDC, 1998). The risk of getting HCV infection through sexual intercourse and household and mother to child during pregnancy is however low compared to other factors (Villena, 2006). The presence of HCV RNA in serum indicates the presence of active infection and a potential for transmission of the infection and/or the development of chronic liver disease. Currently there is no vaccination against hepatitis C. One reason being that the virus comes in many forms and constantly mutates leading to swarms of closely related viral genomic sequences (Abrignani, Houghton, & Hsu, 1999).

2.5. Screening and Diagnosis of Hepatitis C Virus Infection

Screening and diagnosis of Hepatitis C Virus infection involve detection and confirmation of HCV in serum/plasma of infected persons using various serological and molecular assays as well as the assessment of the severity of liver disease (Daw, 2014) (Daw, 2014). Assays based on serology are used to screen anti-HCV antibodies in serum samples. According to CDC, HCV screening is recommended to injecting drug users (IDUs), people who received clotting factor concentrates produced before 1987, those who were on long-term hemodialysis, with persistently abnormal alanine aminotransferase levels (ALT), people living with HIV, recipients of transfusions or organ transplants, including persons who were notified that they received blood from a
donor who later tested positive for HCV infection, who received a transfusion of blood, blood components, or an organ transplant before July 1992. Also is recommended to Healthcare, emergency medical, and public safety workers after needle sticks, sharps, or mucosal exposures to HCV-positive blood, to Children born to HCV-positive women (CDC, 2015). However, it is advised that further confirmation with a second serological assay such as Recombinant Immunoblot Assay (RIBA) be made for any anti-HCV positive samples before molecular based diagnosis is performed.

2.6. Serological Antibody Assays
The first test to be done in HCV screening to see whether the anti-HCV antibodies are present in serum is HCV Antibody testing. The Antibody testing is reported as reactive (positive) or non reactive (negative) (Getchell et al., 2013). In some cases the anti-HCV antibodies can be detected while a person has already cleared the HCV within 6 months or it can be real HCV infection. Over the years, three generations of anti-HCV tests have been developed, with each generation having an improvement in sensitivity and specificity for detecting HCV antibodies (Abdel-Hamid et al., 2002; Filice et al., 1993). Enzyme Immuno Assays (EIAs) and rapid diagnostic tests (RDTs) are currently the most popular serological assays employed to screen blood donors for anti-HCV antibodies or HCV antigen detection (Hans & Marwaha, 2014). They are also used as the preliminary assays in clinical diagnosis of HCV infection. RDTs is performed at room temperature without the use of instrument and it requires serum or plasma, but also fingerstick, capillary whole blood or, for some instances, oral (crevicular) fluid, facilitating screening without the need for venous puncture. Of the three EIA
generations, the second-generation enzyme immunoassay (EIA-2) for antibodies to HCV (anti-HCV) is presently the most commonly used screening test for HCV infection (Daw, 2014). Apart from its qualitative use, EIA is also designed to measure the concentration of antibody in blood or plasma. It has five basic steps: 1) plate wells are coated with antigen; 2) all unbound sites are blocked to prevent false positive results; 3) then primary antibody is added obtained for instance from rabbit monoclonal antibody to the wells; 4) followed by adding secondary antibody conjugated to an enzyme for example anti-mouse IgG; 5) finally a substrate is added to the reaction with the enzyme to produce a color, thus indicating a positive reaction (CDC, 2002).

The Centers for Disease Control and Prevention (CDC) recommends that all positive antibody tests be followed by an HCV RNA test that detects viral RNA in the blood to determine whether or not the person has an active infection because it is believed that HCV RNA will remove this confusion (Alter, Kuhnert, & Finell, 2003). However, diagnosis of HCV by EIA can also be confirmed by RIBA but qualitative RT-RNA PCR confirmation has been found to be sufficient and easier (Alter et al., 2003).

2.7. HCV Nucleic Acid Testing

Antibody assay techniques lack utmost specificity and sensitivity in differentiating between active and resolved infections. As such, for effective diagnosis of HCV infection, molecular assays are highly indispensible in the management of HCV infection (Alter et al., 2003). They are recommended for confirmation of HCV RNA in cases where patients are Seropositive. It is also used for confirmation of presence of HCV viremia in patients who are seronegative but immunocompromised such as HIV.
infected individuals, in babies who are born to HCV positive mothers as antibody in babies can give false positive results up to 18 months of age and for determining the baseline value before starting anti-viral treatment. Both qualitative and quantitative (viral load) molecular assays have been developed in the diagnosis of acute and chronic infection (Firdaus, Saha, Biswas, & Sadhukhan, 2015). They are based on PCR technique whereby targeted regions of viral ribonucleic acid or deoxyribonucleic acid (DNA) are amplified and detected earlier than the other screening methods thus, narrowing the window period between hepatitis C virus (HCV) infections and antibody detection (Hans & Marwaha, 2014). Despite the numerous advantages of NAT, in developing countries, these techniques are underutilized mainly due to financial constraints and lack of technical know-how.

2.8. Molecular Characterization of HCV Genotypes

The methods of identifying and classifying unknown genotypes in clinical samples are rapidly advancing. This method is molecular-based (Cavalheiro, 2007; Mlambo & Kumar, 2008) and the regions of the viral genome most commonly considered are the core, E1, NS4, NS5 and the 5'UTR regions (Gao, 2012; Laperche et al., 2005). However, between the two methods, identification by molecular-based method is considered as the most direct method of which there are three techniques based on sample source. They are; sequencing, hybridization with genotype-specific probes, and real-time polymerase chain reaction (PCR) (Chevaliez & Pawlotsky, 2007; Laperche et al., 2005; Strassl et al., 2015). However, characterization by sequencing followed by phylogenic analysis is regarded as the most reliable technique and the core region is the most preferred
reference region used in many studies for classification of HCV into genotypes and subtypes. The preference for this region over other regions of the viral genome such as 5’UTR and NSB5 is due to the sufficient genetic diversity and the ability to produce topologically identical trees.

2.9. Haematological Abnormalities and HCV Infection

Haematological abnormalities such as anemia, neutropenia, leukopenia, and thrombocytopenia have been reported as major side effects of peg-interferon/ribavirin treatment in HCV patients (Maddrey, 1999). Anemia resulting from treatment is mostly due to ribavirin effect on RBC (Sulkowski, 2005). Whereas ribavirin depletes adenosine triphosphate (ATP) inside red blood leading to oxidative stress and hemolysis, peg-interferon contributes to anemia by bone marrow suppression (Sulkowski et al., 2010; Sulkowski, 2005). Neutropenia can also be induced by antiviral therapy but its occurrence has been found to be associated with dosage (Franciscus, 2013; Sheehan, Weir, & Waters, 2013). It can therefore been managed by dose reduction of interferon-based therapy. However, since maintaining maximum dose of interferon-based therapy is critical to achieving SVR, sever cases of neutropenia can be managed by use of growth factors (Franciscus, 2013). Another hematological toxicity of interferon-based treatment is thrombocytopenia; the most common hematological disorder associated with HCV infected patients (Kedia et al., 2014). Drug-induced thrombocytopenia is usually due to bone marrow suppression, immune-mediated destruction, and platelet aggregation (Wazny & Ariano, 2000). They can range from mild to severe cases following treatment (Li, Han, & Lu, 2010). Although hematological manifestations are
mainly found in patients undergoing treatment, other studies show that, these hemotologic disorders can also be induced by HCV infection in naive-treated patients as a result of extrahepatic manifestations (Chao et al., 2001; Elhajj, Sharara, & Taher, 2004; Spivak, 2000; Streiff et al., 2002). In one study, the frequency of anemia (41.3%), neutropenia (16.6%) and thrombocytopenia (22.6%) was reported among peripheral blood of chronically active HCV infected patients (Khan, Aziz, & Ghazanfar, 2013).

Erythrocyte Sedimentation Rate is a nonspecific measure of inflammation that is commonly used as a medical in vitro screening test (Sox & Liang, 1986). When anticoagulated blood is placed in a vertical column the RBCs normally settle quite slowly. This occurs for 2 main reasons: (1) RBCs repel each other due to the negative charges on their surfaces, or zeta potential, and (2) the large surface-area-to-volume ratio of normal RBCs resists settling. The aggregation of RBCs moves slowly under normal conditions and faster in abnormal conditions. The ESR may be elevated by other conditions that decrease the zeta potential or the RBC surface-area-to-volume ratio. The zeta potential is reduced by other plasma proteins, including immunoglobulin, cholesterol, phospholipids, infectious diseases, neoplastic, invasive tumors, anemia, Chronic and acute diseases of liver by creating more space between RBCs. Decreases in the surface-area-to-volume ratio, as in macrocytosis, also increase the ESR. It decreases by conditions that interfere with the formation of rouleaux or increase the RBC surface-area-to-volume ratio (World Health Rankings, 2015). ESR is measured within a specified time interval usually 1 hour and considered as normal when is less than
20mm/hour and abnormal when is above 20mm/hour using Wistengren method (Shelat, Chacosky, & Shibutani, 2008).

2.10. HCV Treatment Strategies

HCV infection is a killer disease. Without effective treatment interventions, significant increases in CHC associated morbidity and mortality may result. The HCV treatments are now evolving and treatments available are either based on combination therapy or monotherapy. However, the later has been found to be more effective than the former. Thus, most current drugs in used are based on combination therapy and are either interferon-based or interferon-free. Initiation of treatment is based on multiple factors such as liver function (serum bilirubin < 1.5 m g/ dl; albumin > 3.0 g / dl; and no evidence of hepatic encephalopathy or ascites, along with adequate hematological and biochemical parameters to tolerate therapy (hemoglobin > 12 g / dl; neutrophil count > 1.5 k / mm 3; platelet count > 75 k / mm 3; serum creatinine < 1.5 mg / dl). However, HCV genotype and pre-treatment viral load are two significant independent prognostic indicators of antiviral therapy response (Chevaliez & Pawlotsky, 2007; Pawlotsky, 2014). Therefore, a successful treatment regimen is one that leads to high SVR rates and this is dependent of genotype and viral load. For HCV genotype 1, ledispasvir-sofosbuvir and sofobuvir-velpatasvir are two most common interferon-free treatment regimens used for treatment-naïve or treatment-experienced patients due to high SVR rates obtained (Chopra & Muir, 2017). For instance, in an open-label, randomized, phase 2 trial, it was shown that a ledispasvir-sofosbuvir fixed-dose combination treatment of naïve patients for 12 weeks led to SVR rates of more than 95% (Lawitz et al., 2014).
Unlike ledispasvir-sofosbuvir, a 12 week treatment with sofosbuvir-velpatasvir resulted in high rates of SVR among a broad range of patients infected with genotype 1, 2, 4, 5, or 6 (Feld et al., 2015). For genotype 2, apart from treatment with sofosbuvir-velpatasvir, daclatasvir-sofosbuvir regimen is recommended for patients with compensated cirrhosis (Spach & Kim, 2016). Despite its high prevalence rate in the world, HCV genotype 3 is the most treatment-resistant genotype (Ampuero, Romero-Gomez, & Reddy, 2014).

Pegylated interferon (PEG-IFN) alfa combined with ribavirin use to be the only regimen available for achieving SVR in about 65% of patients (Pol, Vallet-Pichard, & Corouge, 2014). However, daclatasvir plus sofosbuvir has recently been approved for treatment of HCV genotype 3 patients. This new regimen has an overall SVR rates of 89% in both treatment-naïve and treatment-experienced patients (Nelson et al., 2015).

2.10.1. Interferons

Interferons (IFNs) are proteins produce normally by cells in body to fight against infection. They work by activating specific genes, influencing cell growth and division, as well as modulating some immune activities (Feld & Hoofnagle, 2005). Thus, IFNs have an indirect antiviral effect on HCV (Peters, 1996). They are in three forms namely; interferon alpha (α), beta (β), and gamma (γ) and they are classified into two group: type I (alpha and beta (β)) and type II (gamma (γ)) depending on the cells by which they act on (Goodbourn, Didcock, & Randall, 2000). They are also produced commercially into two distinct subtypes called IFN alfa-2b (Intron A) and IFN alfa -2a through DNA recombinant techniques and they differ in only 23rd amino acid position where IFN-α 2a has a lysine at that position, whereas IFN-α 2b has an arginine (Pestka, 2000). The
absorption of IFN-α (2a or 2b) is high (above 80%) when administrated intramuscularly or subcutaneously. The concentration typically peaks at 3-12 h after administration. The metabolism and elimination of IFN-α occurs principally via the kidneys, with a half-life of 3-8 hours. Interferons have the derivatives, Peginterferon alfa-2a (Pegasys) and peginterferon alfa-2b (PegIntron, Sylatron) which are produced commercially through the binding of an inert molecule of polyethylene glycol to the recombinant IFN-á and b. Those modified interferons exhibit reduction of renal clearance, altering the metabolism and increasing the half-life of the IFN molecule, although maintaining all of its immuno-stimulatory characteristics (Reddy et al., 2001).

2.10.2. Protease inhibition

Protease inhibition drugs are HCV treatments that act by interrupting posttranslational processing whereby blocking the catalytic site or blocking NS3/NS4A interaction (Pockros, 2010). They are taken orally and include telaprevir boceprevir and paritaprevir. The first reported human study showing a profound and rapid reduction in viral load using a protease inhibitor was in 2003 (Lamarre et al., 2003) and these medications are not taken alone; they are mostly taken in combination with other HCV infection therapies. Both medications are recommended to be taken with food (Chae, Park, & Youn, 2013).
2.10.3. Polymerase Inhibitors

Polymerase Inhibitors are drugs that are also used in treating HCV infection. There are two classes of Polymerase inhibitor drugs; these are nucleoside/nucleotide analogues and non-nucleoside inhibitors. The nucleoside/nucleotide analogues include NM283, R1626, and R7128 and they act by targeting the catalytic sites of the enzyme and act as chain terminators which prevent replication of virus (Chae et al., 2013). Non-nucleoside inhibitors such as HCV-796, ABT-072/333, and GS-9190 are allosteric inhibitors. They are taken in combination with other Hepatitis therapeutic such as pegylated interferon and ribavirin to provide potential suppression of hepatitis C virus RNA. These drugs are NS5B RNA polymerase inhibitors as NS5B enzyme is a highly conserved structure across all hepatitis C genotypes. It is, therefore, an ideal target for drug therapy (Pockros, 2010).

2.10.4. Multi-Class Combination Drugs

Multi-Class Combination Drugs are a combination of drugs formulated into a single pill or package of pills. Harvoni is one example of such drugs presented in combination of two drugs. It consists of the combination of ledipasvir and sofosbuvir which provides the option of administering an interferon-free therapy (Colombo, 2015). Sofosbuvir is a nucleotide analogue HCV NS5B polymerase inhibitor which has been found effective against HCV genotypes 1, 2, 3, 4, and 6 (Gentile, Borgia, Buonomo, Castaldo, & Borgia, 2013; Jacobson et al., 2013). Ledipasvir is an HCV NS5A replication complex inhibitor that interferes with protein that HCV uses to regulate replication as well as virus assembly (Kwon et al., 2015; Link et al., 2014). Harvoni has been found effective in
treating HCV infected adult patients with genotype 1 or 4 and for some people with genotype 3. It can also be used for patients that are treated for the first time or that have had the treatment failure for the previous HCV treatment (Gentile et al., 2013; Gilead Sciences, 2015; Jacobson et al., 2013). For effective treatment to be achieved, diagnosis of genotypes and subtypes need to be known. The reports on prevalence of HCV in Rwanda are limited and those available are only for specific groups. In addition, the genotypes and subtypes circulating have not been investigated.
CHAPTER THREE

3.0. MATERIALS AND METHOD

3.1. Study design

The study was a cross sectional cohort study. The study was designed to enroll both out-patients and in-patients referred for laboratory diagnosis between March, 2016 and June, 2016.

3.2. Study site

The study was carried out at the Rwanda Military Hospital (RMH), one of the referral and teaching hospitals in Rwanda. Rmh is located at Kanombe in Kicukiro district of Kigali province and provides health care services to both military personnel and civilians. It currently treats 80% civilian and 20% military patients (Figure 3.1).

Figure 3.1: Administrative map of Kicukiro district showing the RMH at Kanombe.

3.3. Study Participants and Sample size Estimation

Since RMH is a national referral hospital, the participants of the study were drawn from many parts of the country. The study included both males and females who are above the age of 18 years. Using 3.5% average prevalence of HCV from two similar local studies (Kateera et al., 2015; Rusine et al., 2013), the sample size was estimated using the Kish Leslie formula for cross-sectional studies:

\[ n = \frac{Z^2pq}{d^2} \text{ Eqn(1)} \]

Where \( n \) is the estimated sample size and \( Z \) is the standard normal deviation usually set at 1.96, which corresponds to the 95% confidence interval. \( p \) is the prevalence of HCV derived from previous studies, which is 3.5%. \( q \) is complementary proportion equivalent to one minus \( p \); that is, \( 1 - 0.035 \) equal to 0.965. \( d \) is the degree of absolute precision. A relative precision of 2% (0.02) was used to achieve \( n \) in estimate of HCV prevalence at 95% (Kish, 1965).

Thus,

\[ n = \frac{(1.96)^2 \times 0.035 \times 0.965}{(0.02)^2} = 324 \text{ Eqn(2)} \]

A total of 324 participants were therefore included in the HCV prevalence study.

3.4. Inclusion Criteria

Patients above 18 years reporting to the hospital were included in the study.
3.5. Exclusion Criteria

The study excluded patients below 18 years reporting to the hospital as well as those who did not consent.

3.6. Sampling and Recruitment of Study Participants

Participants recruited into this study were randomly selected and given information about the study. Those who consented to the study were interviewed using a structured questionnaire. Since enrolment of participants took place within two months, the estimated 324 sample size was divided into 162 per month. The 162 was further divided into 9 samples per day. So within a month, 9 randomly selected persons were recruited into the study every day until the 162 sample size was reached for that month.

3.7. Questionnaire Survey

A structured questionnaire to obtain biodata and exposure risks such as blood transfusion, living with HCV infected persons, hospital admission, previous surgery, accidental needstick injuries, treated by traditional doctor, and travelling outside were also administered.

3.8. Blood sample collection

Following recruitment and capturing of all bio-data on the questionnaire during the above step, consented participants were assigned a unique identification number and then directed to a designated trained phlebotomist at a blood collection point. Twenty microlitres of blood was collected by venipuncture into each of two ethylenediaminetetraacetic acid (EDTA) tubes at 10mL each. The blood was mixed by
inverting the tubes immediately after collection and then placed on haematology mixer until processing.

3.9. Anti-HCV Antibody Screening

Blood in one of the EDTA tubes was processed immediately by centrifuging at 3,000 RPM for 5 min to separate plasma. Plasma obtained was screened for anti-HCV antibody using Cypress Anti-HCV dipstick (Cypress diagnosis, Belgium). Cypress Anti-HCV dipstick is an immuno-chromatographic assay for the qualitative detection of hepatitis C antibodies in serum or plasma. This diagnostic assay for HCV antibody testing has been shown to perform with a sensitivity of 95.29% and specificity of 98.75% in previous trial studies. Following manufacturer's instructions, the dipstick was removed from the container and then labeled with participant's identification number. After labeling, the dipstick was immersed into the sample for 15 seconds with the arrow end pointing towards the specimen solution. After 15 seconds, the dipstick was removed and placed flat on a clean surface. Results were read and recorded as negative or positive after 15 minutes. Positive anti-HCV antibody plasma samples were aliquoted at 2 mL in two cryovials and stored at -80°C for viral load measurement, HCV RNA extraction, reverse-transcription, amplification and then sequencing.

3.10. Determination of HCV prevalence

Both active HCV prevalence and anti-HCV antibody prevalence was determined using the formula:

\[
\text{Prevalence} = \frac{\text{Number of Participants with HCV infection}}{\text{Total number of study participants}} \times 100\%
\]
3.11. Hematological and ESR examination

Blood samples (fresh) of all anti-HCV antibody positive samples were subjected to hematological analysis. Cell count, and differential counts were measured using Sysmex XS800i automatic analyzer (Sysmex Corporation, 2006). Blood samples were taken from the hematology mixer. The cap of the tube was removed and the probe of the analyzer inserted into the tube to aspirate about 10µl of blood. Results were then printed out after 3 minutes.

For erythrocyte Sedimentation Rate (ESR), 2mL of whole blood was drawn into Westergren-Katz tube. The tube was then placed in rack in vertical position for 1 hour at room temperature. The distance of fall of erythrocytes was read and recorded in mm/hr (normal range: less than 20mm/h and abnormal above 20mm/h) (Shelat et al., 2008). The remaining blood was centrifuged at 3,000RPM for 5min to separate plasma which was aliquoted at 2 mL in two cryovials and stored at -80°C for viral load measurement and genotyping later.

3.12. Viral Load Measurement

To determine viral load of all seropositive samples (52), one vial of cryopreserved plasma was retrieved from -80°C freezer. After thawing at room temperature for 15min, viral load measurement was performed using Cobas AmpliPrep/Cobas TaqMan HCV machine, version 2 (CAP/CTM HCV v2.0, Roche). Following manufacturer's instructions, the daily maintenance of the equipment (Cobas AmpliPrep instrument and the Cobas TaqMan 96 analyzer) to ensure proper site operation was first carried out. After the equipment was cleaned, the HCV RNA was extracted from 650µL plasma
samples using the Cobas AmpliPrep automated extractor. This was done by loading 650μl of each sample into sample tubes suitable for use in Cobas AmpliPrep /Taqman 96 analyzer. The Cobas AmpliPrep instrument was then started using AMPLILINK software. After RNA extraction, the samples were automatically transferred into Cobas TaqMan 96 analyzer which used to perform reverse transcription, amplification and detection of PCR products. At the end of the detection process, samples with detected RNA were displayed as target detected whereas samples with no detected RNA were displayed as target not detected. Their viral loads were also displayed in international units per milliliters (IU/ml). The IU is an arbitrary amount of a substance agreed upon by scientists and doctors. The results obtained were used to assess viral load of each patient.
3.13. Molecular characterization of the HCV genotypes and subtypes

3.13.1. HCV RNA extraction

To identify and classify the genotypes and subtypes, samples with detectable RNA (N=31) were subjected to manual RNA extraction using QIAamp Viral RNA Mini kit according to the manufacturer’s instructions (Qiagen, Germany) (RNA extracted previous at 3.12 could not be retrieved). Using a pipette, 140µl of plasma was added to 560µl of viral lysis buffer dissolved with 5.6µl carrier RNA (1µg/ml). The resultant mixture was gently vortexed to mix completely for 15min. The tube was then incubated at room temperature (15–25°C) for 10 minutes. After incubating for 10min, tube was then centrifuged to remove drops from the inside of the lid followed by adding 560 µl of ethanol (96–100%) to allow precipitation of RNA. The mixture was then vortexed for 15 sec and centrifuged to remove drops from inside the lid. Six hundred and thirty microlitres of supernatant was transferred into the QIAamp spin column in a 2-ml collection tube and then centrifuged at 6000 x g (8000 rpm) for 1 min. After centrifugation the QIAamp spin column was placed into a clean 2-ml collection tube, and the tube containing the filtrate discarded. Five hundred microlitres of two wash buffers AW1 and AW2 were added in succession with 1and 3 minutes of centrifugation at 6000 x g and 20,000 x g, respectively. After washing, the RNA bound on the column was then eluted into 1.5ml micro centrifuge tube by addition of 200µl of elution buffer AVE followed by incubation at room temperature for 1 min and a final centrifugation at 6000 x g(8000 rpm) for 1 min. After centrifugation, the extract was kept at -80°C for Reverse transcription-polymerase chain reaction (RT-PCR amplification).
3.13.2. Reverse Transcription and Polymerase Chain Reaction

RNA extracts were retrieved from -80°C storage and kept on ice to thaw. Using one-step RT-PCR kit (Qiagen, Germany), reverse transcription of RNA into complementary DNA (cDNA) was performed and amplification of cDNA were carried out in a single step on 24 of the samples and one-step RT-PCR kits, reverse transcription on 7 samples was done using QuantiTect Reverse transcription kit (Qiagen, Germany). First, only two samples were reverse transcribed with QuantiTect Reverse transcription kit and amplified. After confirming on 1% gel, the QuantiTect Reverse transcription kit was used for the remaining 5 samples. The reaction was made up of the following components; 6µL RT-PCR buffer, 1.2µL dNTP Mix (400µM of each dNTP), 1.2µL RT-PCR enzyme mix, 9.4µL RNase-free water, 0.6µL MgCl₂, 1.8µL (0.6µM) outer forward primer (5'-ACT GCC TGA TAG GGT GCT TGC-3'), 1.8µL (µM) outer reverse primer (5'-ATG TAC CCC ATG AGG TCG GC-3') and 8.0µL RNA template to make 30µL reaction volume. The primers used in this reaction target a 405nt of HCV core region (Lole et al., 2003). The RT-PCR conditions were as follows; reverse transcription for 30 minutes at 50°C; initial PCR activation step for 15 minutes at 95°C; three-step cycling for 35 cycles which involve, denaturation for 1 minute at 94°C, annealing for 1 minute at 59°C and extension for 1 minute at 72°C and final extension for 10 minutes at 72°C. Concentration of the RT-PCR product was determined using QIAxpert system (Qiagen, Germany) and subsequently diluted to make a concentration of 8.66ng/µL (identified through optimization) in a 30µL PCR reaction volume for inner PCR system. The PCR reaction consisted of the following components; 15µL HotStarTaq Plus Master, 0.6µL
MgCl₂, 2.4µL RNase-free water, 1.5µL (0.5µM) each of inner forward and reverse primers and DNA template at 8.66ng/µl. The inner forward primer (5'-AGG TCT CGT AGA CCG TGC A-3') and inner reverse primer (5'-CAC GTT AGG GTA TCG ATG AC-3') used targets 360 nucleotides of the HCV core region (Lole et al., 2003). The PCR conditions were as follows; denaturation for 1 minute at 94°C, annealing for 1 minute at 60°C and extension for 1 minute at 72°C and final extension for 10 minute at 72°C in three-step cycling for 35 cycles.

3.13.3. Gel Electrophoresis and Visualization

PCR products of all 31 reactions were confirmed on 1% agarose gel and visualized under ultraviolet light. The gel was prepared by weighing 1.0g of agarose into 100mL of 1X Tris Borate EDTA buffer. The slurry was heated in a microwave until the agarose dissolved. The solution was then allowed to cool to 60°C. Ethidium bromide (5µL) was added and mixed thoroughly. The gel was then poured onto a tray fixed with a well comb and allowed to polymerize for 30 minutes. The tray together with the solidified gel was placed in an electrophoresis tank. About 800 mL TBE buffer was then poured into the tank to cover the gel. The comb was then removed. Five microlitres of PCR product (DNA) was mixed with 2 µl of gel loading dye (Bromophenol blue) and then loaded slowly into the wells of submerged gel. A 1kb DNA molecular weight marker (Gelpilot® Plus) (Qiagen, Germany) at 6 µL was loaded into two extreme wells to enable size estimation of the resolved bands. The gel was run under constant voltage of 110 for 1 hour. After 1 hour, gel was visualized under UV light. After confirmation on
gel, samples were submitted to Macrogen Europe Laboratory (Macrogen, Netherlands) for purification and sequencing.

3.13.4. Purification of PCR Products

Purification of the second PCR products was done using ExoSAP clean up protocol. ExoSAP is a combination of two enzymes; Exonuclease I and Shrimp Alkaline Phosphatase. The exonuclease I removes leftover primers, while the Shrimp Alkaline Phosphatase removes any remaining dNTPs. Following manufacturer's protocol, five microlitres of post-PCR reaction product was added to 2 μl of ExoSAP-IT for a combined reaction volume of 7 μl. The mixture was incubated at 37°C for 15 minutes to degrade remaining primers and nucleotides. This incubation was followed by a second incubation at 80°C for also 15 minutes to inactivate the two enzymes.

3.13.5. Sequencing of Purified DNA

Purified products were sequenced in DNA Engine Tetrad 2 Peltier Thermal Cycler (BIO-RAD, USA) and analyzed using ABI 3730xl DNA Analyzer (Applied Biosystems). The BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems) was used. Briefly, Sequencing reactions were performed in the DNA Engine Tetrad 2 Peltier Thermal Cycler using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit, following the protocols supplied by the manufacturer. Single-pass sequencing was performed on one template using the sequencing primer, 5’-AGG TCT CGT AGA CC G TGC A -3’. The fluorescent-labeled fragments were purified from unincorporated terminators with the BigDye XTerminator® Purification Kit (Applied
Biosystems) according the manufacture protocol and then the samples were injected to electrophoresis in an ABI 3730xl DNA Analyzer (Applied Biosystems) to finalize the sequencing. The sequence of the 31 sample was provided and analysed.

3.13.6. Genotyping and Phylogenetic Analysis

Phylogenetic analysis of a 360-bp region of the HCV core region was used to determine HCV genotypes. A hundred and eighty-sixty genotype genomic references were obtained from HCV sequence database (Kuiken, Yusim, Boykin, & Richardson, 2005), Alignment ID : C14RG1. The genomic sequences were trimmed using BioEdit Sequence Alignment Editor (Ibis Biosciences) to have the 5’ non-coding region (NCR). The sequences were then aligned with the 31 sequenced HCV cDNA samples using MUSCLE as implemented in MEGA 7.0. 14 software (Tamura et al., 2011) and a phylogenetic tree made using the same software. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 2011). The tree with the highest log likelihood (-2945.3336) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.3338)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 43 nucleotide sequences. Codon positions included were
1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 340 positions in the final dataset.


All data were analyzed using SPSS version 16.0, Comparison between categorical variables was computed using Fisher’s exact test. A p value < 0.05 was considered statistically significant

3.15. Ethical Approval

Ethical approval was obtained from the Rwanda Military Hospital.
CHAPTER FOUR

4.0. RESULTS

4.1. Demographic Characteristics of Study Participants

A total of 324 patients referred for laboratory diagnosis were randomly selected to participate in the study which was conducted from April to June 2016. Of the 324 study participants, 133 (41.0%) were males and 191 (59.0%) were females. The average age of the population was 42.32 years. The youngest participant was 18 years whereas the oldest participant was 89 years. The characteristics of the study population defined by various demographic variables are shown in Table 4.1.

Table 4.1: Demographic Characteristics of Participants in the Study

<table>
<thead>
<tr>
<th>Variable</th>
<th>Number of Participants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>133 (41.0%)</td>
</tr>
<tr>
<td>Female</td>
<td>191 (59.0%)</td>
</tr>
<tr>
<td>Age group (yrs)</td>
<td></td>
</tr>
<tr>
<td>18-35</td>
<td>123 (38.0%)</td>
</tr>
<tr>
<td>36-55</td>
<td>134 (41.4%)</td>
</tr>
<tr>
<td>&gt;55</td>
<td>67 (20.7%)</td>
</tr>
<tr>
<td>Marital Status</td>
<td></td>
</tr>
<tr>
<td>Single</td>
<td>73 (22.5%)</td>
</tr>
<tr>
<td>Married</td>
<td>207 (63.9%)</td>
</tr>
<tr>
<td>Divorced</td>
<td>37 (11.4%)</td>
</tr>
<tr>
<td>Widow</td>
<td>7 (2.2%)</td>
</tr>
<tr>
<td>Employment Sector</td>
<td></td>
</tr>
<tr>
<td>Commerce</td>
<td>71 (21.9%)</td>
</tr>
<tr>
<td>Category</td>
<td>Count</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Health</td>
<td>13</td>
</tr>
<tr>
<td>Agriculture (farming)</td>
<td>46</td>
</tr>
<tr>
<td>Security</td>
<td>35</td>
</tr>
<tr>
<td>Transport</td>
<td>8</td>
</tr>
<tr>
<td>Education and Religion</td>
<td>44</td>
</tr>
<tr>
<td>Technical Staff</td>
<td>15</td>
</tr>
<tr>
<td>Unemployed</td>
<td>73</td>
</tr>
<tr>
<td>Other</td>
<td>19</td>
</tr>
<tr>
<td>Place of Birth</td>
<td></td>
</tr>
<tr>
<td>Kigali</td>
<td>47</td>
</tr>
<tr>
<td>Southern</td>
<td>99</td>
</tr>
<tr>
<td>Northern</td>
<td>33</td>
</tr>
<tr>
<td>Eastern</td>
<td>40</td>
</tr>
<tr>
<td>Western</td>
<td>28</td>
</tr>
<tr>
<td>Abroad</td>
<td>77</td>
</tr>
<tr>
<td>Place of Residence</td>
<td></td>
</tr>
<tr>
<td>Kigali</td>
<td>240</td>
</tr>
<tr>
<td>Southern</td>
<td>26</td>
</tr>
<tr>
<td>Northern</td>
<td>23</td>
</tr>
<tr>
<td>Eastern</td>
<td>29</td>
</tr>
<tr>
<td>Western</td>
<td>6</td>
</tr>
<tr>
<td>Level of Education</td>
<td></td>
</tr>
<tr>
<td>Primary</td>
<td>118</td>
</tr>
<tr>
<td>Secondary</td>
<td>119</td>
</tr>
</tbody>
</table>
4.2. Prevalence of Hepatitis C Virus Infection among Study Participants

4.2.1 HCV Seroprevalence among Study Participants

Anti-HCV antibodies were detected in 52 out of the 324 samples making an overall seroprevalence of 16.0%. Seroprevalence in males was higher (18.0%) than in females (14.7%), although the difference was not statistically significant (p = 0.444; \( \chi^2 = 0.667 \)) table 4.2. There was however, a significant difference (p<0.05; \( \chi^2 = 42.948 \)) in HCV seroprevalence among the age groups with HCV infection increasing significantly with age. Detection of HCV antibodies was most prevalent among older age group (above 55years) and least among younger age groups (Table 4.3).

4.2.2 Active HCV RNA Prevalence among seropositive Participants

Confirmation of active HCV RNA infection among seropositive participants revealed that, of the 52 anti-HCV positive participants, 31 had detectable viremia whereas 21 were viral load negative. Thus, an overall active HCV prevalence of 9.6% (31/324) was recorded (Figure 4.1).
The prevalence of anti-HCV antibodies and HCV RNA in terms of gender as determined by the combination of immuno-chromatographic strips and real time PCR procedures is shown in Table 4.2. There was no significant difference between prevalence of active HCV RNA ($p=0.916$, $\chi^2=0.011$) and gender.

**Table 4.2:** Gender wise distribution of anti-HCV antibodies and HCV RNA

<table>
<thead>
<tr>
<th>Gender</th>
<th>No. of Participants</th>
<th>Anti-HCV +ve</th>
<th>HCV RNA +ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>133</td>
<td>24 (18.0%)</td>
<td>13 (9.8%)</td>
</tr>
<tr>
<td>Female</td>
<td>191</td>
<td>28 (14.7%)</td>
<td>18 (9.4%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>324</strong></td>
<td><strong>52 (16.0%)</strong></td>
<td><strong>31 (9.6%)</strong></td>
</tr>
</tbody>
</table>
The participants were divided into three age categories. Out of the 324 participants tested with immunochromatographic strips, anti-HCV antibodies were detected in 41.8% participants older 55 years, 11.9% middle age participants (36-55 years) and 6.5% younger participants (18-35 years). In general, it was observed that, prevalence of active HCV infection increased significantly with increasing age (p=0.001). The age wise Prevalence of anti-HCV antibodies and active HCV RNA (Table 4.3).

Table 4.3: Age-wise Prevalence of anti-HCV antibodies and HCV RNA among study participants

<table>
<thead>
<tr>
<th>Age Group(yrs)</th>
<th>No. of Participants</th>
<th>Anti-HCV +ve</th>
<th>HCV RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-35</td>
<td>123</td>
<td>8 (6.5%)</td>
<td>3 (2.4%)</td>
</tr>
<tr>
<td>36-55</td>
<td>134</td>
<td>16 (11.9%)</td>
<td>9 (6.7%)</td>
</tr>
<tr>
<td>&gt;55</td>
<td>67</td>
<td>28 (41.8%)</td>
<td>19 (28.4%)</td>
</tr>
<tr>
<td>Total</td>
<td>324</td>
<td>52 (16.0%)</td>
<td>31 (9.6%)</td>
</tr>
</tbody>
</table>

Prevalence of active HCV infection was highest among participants born in Southern Rwanda (12.6%; 13/99) and least among participants born outside Rwanda (3.9%; 3/77). However no significant relationship was found between participant's birth place and active HCV RNA infection (p= 0.390, \( \chi^2=5.213 \)). Similarly, active HCV infection prevalence was highest in southern residence (26.9% ;7/26) and least among Kigali residence(7.5%;18/240) In contrast, there was a significant (p = 0.031; \( \chi^2=10.627 \))
relationship between participants place of residence and active RNA infection (Figure 4.2).

**Figure 4.2:** Prevalence of HCV RNA infection according to birth place and Residence.

HCV infection was most commonly found among widows/widowers and least among divorced participants as shown in table 4.4. However, no significant relationship was found (p>0.05).
4.3. Risks Factors of Acquiring HCV Infection

Comparison of risk factors of acquiring HCV infection among participants was also analyzed (Table 4.5). It was observed that participants who have received injection from traditional doctors stand a significant (p=0.036) chance of having HCV infection. Similarly, persons who have lost a relative through hepatitis C (HC) and those with no educational background were more likely to have HCV infection (Table 4.5).
<table>
<thead>
<tr>
<th>Risk Factor</th>
<th>HCV RNA +ve</th>
<th>Percent</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Transfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>2</td>
<td>10.5%</td>
<td>0.939</td>
</tr>
<tr>
<td>No</td>
<td>29</td>
<td>9.5%</td>
<td></td>
</tr>
<tr>
<td>Relative died of HC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>8</td>
<td>8.0%</td>
<td>0.052</td>
</tr>
<tr>
<td>No</td>
<td>21</td>
<td>14.0%</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>33.3%</td>
<td></td>
</tr>
<tr>
<td>Living with infected person</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>7</td>
<td>13.7%</td>
<td>0.271</td>
</tr>
<tr>
<td>No</td>
<td>24</td>
<td>8.8%</td>
<td></td>
</tr>
<tr>
<td>Hospitalized</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>12</td>
<td>7.9%</td>
<td>0.354</td>
</tr>
<tr>
<td>No</td>
<td>19</td>
<td>11.0%</td>
<td></td>
</tr>
<tr>
<td>Surgeries Performed</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.5: Risk/Exposure Factors of HCV Infection among 324 Study Participants
<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>6</th>
<th>8.5%</th>
<th></th>
<th>No</th>
<th>25</th>
<th>9.9%</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Accidental needlestick injuries</td>
<td>Yes</td>
<td>5</td>
<td>26.3%</td>
<td>0.036*</td>
<td>No</td>
<td>26</td>
<td>8.3%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treated by Traditional Doctor</td>
<td>Yes</td>
<td>12</td>
<td>12.9%</td>
<td>0.195</td>
<td>No</td>
<td>19</td>
<td>8.2%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Travelled outside before</td>
<td>Yes</td>
<td>22</td>
<td>11.3%</td>
<td>0.197</td>
<td>No</td>
<td>9</td>
<td>7.0%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Education Background</td>
<td>Primary</td>
<td>15</td>
<td>12.7%</td>
<td>0.097</td>
<td>Secondary</td>
<td>10</td>
<td>8.4%</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tertiary</td>
<td>3</td>
<td>4.1%</td>
<td></td>
<td>None</td>
<td>3</td>
<td>21.4%</td>
<td></td>
</tr>
</tbody>
</table>

*: Significant at 95% confidence interval
4.4. Hematological Profiles of HCV Infected Participants

Haematological abnormalities observed amongst the HCV infected participants were leukocytopenia (48.4%), lymphopenia (3.2%), and neutropenia (6.5%) and thrombocytopenia (25.8%) (Table 4.6). Leukocytopenia was more prevalent but not significant ($p>0.05$) in males (53.8%) than in females (44.4%). In contrast, thrombocytopenia was common in females (33.3%) than males (15.4%). With regard to age, thrombocytopenia was only found in participants above 55 years compared to other age groups ($p=0.033$). Only one female (5.6%) had anemia. Erythrocytes Sedimentation Rate (ESR) was recorded under two categories; $>20$mm/hr and $<20$mm/hr. Overall, 5 (16.1%) HCV patients had ESR values above 20mm/hr, with 23.1% and 11.1% being males and females respectively.

**Table 4.6:** Prevalence of Haematological Abnormalities among HCV Infected Participants.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Gender</th>
<th>Age(yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (n=13)</td>
<td>Female (n=18)</td>
</tr>
<tr>
<td>Leukopaenia</td>
<td>15(48.4)</td>
<td>7(53.8%)</td>
</tr>
<tr>
<td>Lymphopaenia</td>
<td>1(3.2%)</td>
<td>1(7.7%)</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>2(6.5%)</td>
<td>1(7.7%)</td>
</tr>
<tr>
<td>Thrombocytopaenia</td>
<td>8(25.8%)</td>
<td>2(15.4%)</td>
</tr>
</tbody>
</table>
*: Significant at 95% Confidence interval. Computed using Chi-square test. Reference range of hematological variables: Total WBC count (4.50 -11.50[10^3/μL]) Lymphocytes (Female: 1.30-3.70, Males: 1.20-4.80[10^3/μL]); Neutrophils (Females: 1.10-4.40, Males: 2.30-8.10 [10^3/µL]); Platelets (150-540 [10^3/µL]), Haemoglobin (Females: 11.0-17.0, Males: 12.0-18.0[g/dL]) and ESR ( < 20mm/hr) (Sysmex Corporation, 2006).

### 4.5. Hepatitis C Viral Load Measurement

Of the 52 samples testing positive to anti-HCV antibodies, HCV RNA was detected in 31 (59.62%). The viral load ranged from 2.3x10^3 to 2.9x10^7 IU/mL with mean ± SD values of 2.5x10^6 ± 5.2x10^5 IU/mL and a median value of 9.7x10^5 IU/mL. Viral loads recorded were divided into two categories; low viral load (< 800,000IU/mL) and high viral load (≥ 800,000IU/mL) (Alan, 2016). High viral load was detected in 58.1% (18/31) of the participants whereas 41.9% (13/31) recorded low viral load. The viral load levels were not significantly different between age groups and gender (Table 4.7).
Table 4.7: HCV Viral load Measured among HCV RNA Positive Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Viral load (IU/mL)</th>
<th></th>
<th></th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 800,000 (low)</td>
<td>≥ 800,000 (high)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6 (46.2%)</td>
<td>7 (53.8%)</td>
<td>0.686</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>7 (38.9%)</td>
<td>11 (61.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18-35</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36-55</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
<td>0.918</td>
<td></td>
</tr>
<tr>
<td>Above 55</td>
<td>8 (42.1%)</td>
<td>11 (57.9%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4.6. Molecular Characterization of HCV Genotypes and Subtypes in Study Participants

4.6.1. PCR amplification of core region

In order to characterize the HCV genotypes and subtypes circulating in the population, HCV RNA of all 31 samples with active RNA detected were analyzed. In all 31 samples the core region of HCV virus was amplified. The band size was approximately 360bp (Figure 4.3).
Figure 4.3: Gel Image showing bands for A) 24 B) 2 and C) 5 making a total of 31 samples run on 1% Agarose gel. For A, Reverse transcription and PCR were done using one-step RT-PCR kit; For B and C, reverse transcription and PCR was done using QuantiTect Reverse transcription kit. M, Molecular marker(1kb); S1- S31, samples; NS, No sample; NC, negative control. DNA bands on %1 Gel was stained with ethidium bromide and run at 110V for 60min. Visualization was done under UV light.

4.6.2. HCV Genotypes and subtypes

In this study HCV genotype 3 (GT3) and genotype 4 (GT4) were identified. The subtypes of these genotypes identified had 92% to 97% identity to reference isolate obtained from National Center for Biotechnology Information (NCBI). The various subtypes of the genotypes identified are shown in Table 4.8.
**Table 4.8:** BLAST results of the 31 sequences

<table>
<thead>
<tr>
<th>Sample # (s)</th>
<th>Genotype</th>
<th>Subtype</th>
<th>Reference Isolate/Accession #</th>
<th>Percentage Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>S28, S22</td>
<td>3</td>
<td>3h</td>
<td>SOM2/ AF216793</td>
<td>92%</td>
</tr>
<tr>
<td>S17, S8</td>
<td>4</td>
<td>4c</td>
<td>S73421/S73421</td>
<td>97%</td>
</tr>
<tr>
<td>S1 &amp; S2, S16</td>
<td>4</td>
<td>4v</td>
<td>CYHCV073/HQ537009</td>
<td>95%</td>
</tr>
<tr>
<td>S3, S4, S7, S10, S11, S13, S15, S18, S19, S20, S23, S29, S30, S31</td>
<td>4</td>
<td>4k</td>
<td>PS3/EU392173</td>
<td>94%</td>
</tr>
<tr>
<td>S9, S5, S24, S26</td>
<td>4</td>
<td>4q</td>
<td>QC262/FJ462434</td>
<td>97%</td>
</tr>
<tr>
<td>S14, S27</td>
<td>4</td>
<td>4r</td>
<td>SA1686/KC143908</td>
<td>97%</td>
</tr>
<tr>
<td>S25, S21, S12, S6, S16</td>
<td>4</td>
<td>4n</td>
<td>AGJ89972</td>
<td>95%</td>
</tr>
</tbody>
</table>

GT4 was present in 93.5% (29/31) of HCV RNA positive participants whereas only 6.5% (2/31) were genotype 3. The GT3 isolates were both subtype 3h. However, for GT4, there were 7 subtypes identified. The most prevalent subtype of this genotype was 4k (48.3%; 14/29) (**Figure 4.4**).
There was no significant relationship between HCV genotype or subtype and either gender or age. The prevalence of the genotypes and subtypes according to gender and age groups are shown in Figure 4.5.
Figure 4.5: Prevalence of HCV genotype 3 and 4: A and B and subtypes: C and D according to gender and age. Asterisk (black, red and blue) mean no genotype or subtype identified.

Of the 18 HCV RNA positive participants residing in Kigali province, one was infected with GT 3 and 17 were infected with GT4. Also, with the 7 HCV RNA positive participants residing in Southern province, one was infected with GT 3 and six with GT4. Residents of Western and Eastern were infected with only GT 4 (1 and 3 respectively). In contrast, participants born in Kigali were infected with only GT-4 whereas participants born Southern Rwanda were infected both GT- 3 and GT-4 (2 and
11 respectively). All other participants from the remaining provinces of birth were infected with only GT-4. The distribution of HCV subtypes according to residence and birth place of participants is depicted in Figure 4.6.
Figure 4.6: Cases of HCV subtypes according to (A) Place of birth and (B) Place of Residence.
5.6.3. HCV Genotype and Viral Load

Viral load was classified into two categories; low (< 800,000 IU/mL) and high (≥ 800,000 IU/mL). Analysis shows there was no significant relationship between HCV viral load and HCV genotype (Table 4.9).

**Table 4.9:** Prevalence of HCV Genotypes According to Viral Load Categories

<table>
<thead>
<tr>
<th>Genotype/Subtype</th>
<th>N (%)</th>
<th>&lt;800,000</th>
<th>&gt;800,000</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3h</td>
<td>2 (6.5%)</td>
<td>1 (7.7%)</td>
<td>1 (5.6%)</td>
<td>0.811</td>
</tr>
<tr>
<td>4c</td>
<td>2 (6.5%)</td>
<td>1 (7.7%)</td>
<td>1 (5.6%)</td>
<td>0.811</td>
</tr>
<tr>
<td>4k</td>
<td>14 (45.2%)</td>
<td>6 (46.2%)</td>
<td>8 (44.4%)</td>
<td>0.925</td>
</tr>
<tr>
<td>4n</td>
<td>4 (12.9%)</td>
<td>1 (7.7%)</td>
<td>3 (16.7%)</td>
<td>0.462</td>
</tr>
<tr>
<td>4o</td>
<td>1 (3.2%)</td>
<td>0</td>
<td>1 (5.6%)</td>
<td>0.388</td>
</tr>
<tr>
<td>4q</td>
<td>4 (12.9%)</td>
<td>2 (15.4%)</td>
<td>2 (11.1%)</td>
<td>0.726</td>
</tr>
<tr>
<td>4r</td>
<td>2 (6.5%)</td>
<td>1 (7.7%)</td>
<td>1 (5.6%)</td>
<td>0.811</td>
</tr>
<tr>
<td>4v</td>
<td>2 (6.5%)</td>
<td>1 (7.7%)</td>
<td>1 (5.6%)</td>
<td>0.811</td>
</tr>
</tbody>
</table>

*P-value > 0.05 indicates no relationship between HCV viral load and HCV subtypes*
5.6.4. Phylogenetic Analysis

To further verify the genotypes and subtypes, phylogenetic tree was constructed with the following reference sequences; 1a.HQ537007, 2a.AB047639, 3h.JF735126, 4c.FJ462438, 4m.FJ462433, 4r.FJ462436, 4q.FJ462434, 4k.FJ462438, 4v.HQ537009, 5a.NC009826, 6a.EU246930 and 7a.EF108306 (Figure 4.7).
Figure 4.7: Phylogenetic tree for 31 samples from patients for identification of HCV genotypes. The isolates obtained from this study are designated as S1 to S31.
CHAPTER FIVE

5.0. DISCUSSION

5.1. Overview

Viral hepatitis is a major infectious disease of global concern (Stanaway et al., 2016). In sub-Saharan Africa, viral hepatitis due to HCV infection is highly prevalent but the extent of the disease burden may be under reported (Karoney & Siika, 2013; Lemoine, Eholić, & Lacombe, 2015). More importantly, HCV genotyping and assessment of viral load are vital to treatment strategies. Thus, the aim of this study was to estimate the active prevalence of HCV infection in Rwanda and characterize the HCV genotypes and subtypes circulating in the population.

5.2. Seroprevalence of Hepatitis C Virus Infection among Study Participants

Prevalence of HCV infection varies across different regions and populations (Messina et al., 2014). In Africa, prevalence of HCV infection reported so far have focused on specific group of the population mostly relying on the error-prone antibody testing method (Atsbaha et al., 2016; Ikeako, Ezegwui, Ajah, Dim, & Okeke, 2014; Pirillo et al., 2007; Rusine et al., 2013). In this study, participants were first screened for the presence of HCV antibodies using rapid diagnostic strips. Seropositive cases were then subjected to the more sensitive polymerase chain reaction method. A high seroprevalence of 16.0% was reported in patients attending the national referral hospital in Rwanda. This figure is comparatively higher than the 1.3% seroprevalence reported
by Kateera et al., (2015) with the same Cypress Anti-HCV dipstick. This test has 98.7% relative specificity for antibodies against both structural and non-structural proteins of HCV (Eze et al., 2014). However, the assay generally lacks the discriminatory ability to differentiate between antibodies resulting from active infection and previously cleared infection (Seremba et al., 2010). Several studies have shown that, HCV infection is mostly cleared spontaneously before progression into chronic infection during primary infection (Gerlach et al., 2003; Micallef, Kaldor, & Dore, 2006; Rao et al., 2012). Even though the underlining mechanism for such clearance is not well articulated, in one study conducted among Chinese population, female gender, a history of acute icteric hepatitis, serologic evidence of HBV coinfection and the rs12979860 CC genotype were identified as factors responsible for viral clearance (Kong et al., 2014). Individuals with such attributes may usually test false positive to anti-HCV antibody testing (Seremba et al., 2010).

It is therefore recommended that all patients having anti-HCV antibodies are subjected to further confirmatory testing to determine whether the patient has active RNA infection thereby eliminating such false positives (World Health Organization, 2014).

5.3. Prevalence of Active Hepatitis C Virus RNA among Study Participants

In order to ascertain the true state of HCV infection among the study participants, active HCV RNA infection among all seropositive participants was assessed by viral load measurement and direct PCR amplification. Present results show that, of the 52 seropositive participants, 31 (59.62%) had active HCV RNA infection. This translates to
an overall active prevalence of 9.6%. The active prevalence reported in this study is higher compared to 3.7% reported in Democratic Republic of Congo (Iles et al., 2013) and 4.9% reported in Pakistan (Anwar, Rahman, Hassan, & Iqbal, 2013). The prevalence indicates the cost-effectiveness of screening using serological tests and confirmation by RNA. The current cost of RNA testing in Rwanda is about $100.00, limiting the general public to only HCV antibody screening.

This study noted infection was higher in males than in females. Many studies have reported similar findings (Mwangi et al., 2016; Rao et al., 2012). This high infection rate in males than in females may be due to spontaneous clearance of acute infection in females (Micallef et al., 2006). The reason to this clearance has been attributed occurrence of certain genetic factors such as IL28B genetic variants in females (Bakr et al., 2006; Chung, 2005). Similarly, prevalence of active HCV infection increased significantly with age and this is similar to findings reported in Uganda by O’Reilly et al., (2011) and in Madagascar by Ramarokoto et al., (2008) and this may be due to frequent exposure (Abdel-Aziz et al., 2000). With the high prevalence being reported in patients older than 55 years, more screening for HCV can focus on this age group.

5.4. Risk Factors of Acquiring HCV Infection

In developing countries various risk factors associated with acquiring HCV infection have been reported in various studies (Atsbaha et al., 2016; Daw et al., 2014; Jatapai et al., 2010). Identification of risk factor enables appropriate control strategies to be developed. In this study hospital based risk factors were not found to be significant. This is in contrast to a study in Ethiopia where history of hospitalization, tooth extraction
and blood transfusion were identified as major risk factors HCV infection (Atsbaha et al., 2016). Instead, participants who have been injected before by traditional doctors were found to be the group at greatest risk. This observation may be due to the fact that, needles used may be unsterilized and also be used for more than one person. A significant observation made from this study is that, HCV prevalence was highest among persons residing in Southern Rwanda followed by Western Rwanda. The high prevalence of infection in this part of Rwanda may be due to migration of persons at the border with Burundi where prevalence is highest in East Africa (Karoney & Siika, 2013). It is therefore recommended that, more screening for persons living in Southern Rwanda and at various ports of entry be done. The current study showed that HCV prevalence was highest in widows/widower and also in married or once married individuals. This high prevalence may imply that, transmission via sex is important in Rwanda.

5.5. Hematological Abnormalities in HCV Infected Participants

Anemia, neutropenia, leukopenia, and thrombocytopenia are hematological disorders associated with HCV infection especially among those undergoing treatment (Dieterich & Spivak, 2003; Dusheiko et al., 1996; Kedia et al., 2014; Nachnani et al., 2010). However, in some cases, autoimmune hemolytic anemia, leukopenia, and thrombocytopenia may be induced by the infection itself (Srinivasan, 2001). In the present study, anemia, neutropenia, leucopenia and thrombocytopenia were identified among HCV infected participants. However there was a significant association of HCV infection and thrombocytopenia. This observation may be due to autoimmune
mechanisms and decrease in liver thrombopoietin production (Fouad, 2013). Similar findings have been reported by (Olariu, Olariu, & Olteanu, 2010)

5.6. Hepatitis C Viral Load and Genotyping

Assessment of HCV viral load and genotype are critical to initiation of HCV treatment strategies. Low baseline viral load prior to initiation of HCV treatment has positive effect on treatment outcomes in terms of achieving successful sustained virologic response (SVR) (Dalgard et al., 2004; Von Wagner et al., 2005). The most widely accepted HCV RNA level used to define “high” and “low” pretreatment viral load in studies using conventional (non-pegylated) interferon-based therapy has been 800,000 IU/mL (Martinot-Peignoux et al., 2009). In this study, most actively infected participants 58.1% (18/31) had high viral load (>800,000 IU/mL) suggesting an immediate commencement of treatment. However, other factors such as serum alanine aminotransferase levels, hemoglobin levels and neutrophil count have to be determined (Xing, et al., 2012; AASLD/ IDSA, 2015). In this study, it was observed that, of the 18 who had viral load >800,000 IU/mL, 10 had neutrophil count >1500/mm³ and hemoglobin level within the normal range (at least 13g/dl for men or 12g/dl for women). Therefore initiation of treatment of these participants is recommended after their ALT levels have been determined since this parameter was not evaluated. Also, high viral load was common among females and participants older than 55 years. There was no significant correlation between the viral load, age and gender observed. This is in agreement with other studies in which no significant relationship between viral load, age and gender was observed (Afridi et al., 2014; Schijman et al., 2004). Increased HCV
viral load have been reported among patients co-infected with HIV (Matthews-Greer et al., 2001) and other hepatic viruses. However, HIV infection is not investigated in this study.

Globally, Egypt has the highest active prevalence (15%) of HCV with GT-4 accounting for more than 90% infections. Similarly, studies have shown that HCV GT-4 is highly endemic in East and West African countries such as Rwanda and other neighboring countries such as Tanzania and Uganda and Liberia (Kamal & Nasser, 2008). In this study, 29 (93.5%) out of the 31 HCV RNA positive participants were infected with genotype 4 with subtype 4k being the most prevalent. Thus, the findings of this study are similarly to other studies where HCV GT-4 has been reported as the most prevalent (Kamal & Nasser, 2008). Studies have shown that patients with 4 are more likely to have significantly higher fibrosis progression and severe fibrosis cirrhosis than non genotype 4 patients (Wali et al., 2003). The other rare and serious genotype identified was GT-3 belonging to subtype 3h which accounted for only 6.5%. Similarly, HCV genotype 3h is known to be associated with liver steatosis as well as difficult to treat (Negro, 2002).

5.7. Conclusion

The current study shows a high seroprevalence of 16.0% and active prevalence of 9.6% in Rwanda. Infection was common in males than in females with older persons being the most infected group. Hematological abnormalities observed were Leukocytopenia, lymphopenia, neutropenia and thrombocytopenia and were commonly associated with HCV infected males than females. Injection by traditional doctors was a major risk
factor of acquiring HCV infection. HCV genotypes 3 and 4 were detected among actively infected participants with genotype 4 being the most prevalent genotype.

5.8. Recommendations

Looking at the results of the present study, the following recommendation has been made;

i. Activities of traditional doctors must be regulated

ii. Persons having low platelet count should be considered for HCV testing

iii. More screening should be done in males, older persons and persons residing in Western and Southern Rwanda

iv. In order to break the cycle of HCV transmission, it is highly recommended that, screening among widows, widowers and sexually active individuals be encouraged.
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APPENDICES

APPENDIX I: Ethical Approval Form

April 8th, 2016

Ref.: EC/ RMH/ 044/ 2016

REVIEW APPROVAL NOTICE

Dear Umumararungu Esperance
Pan African University

Your research project: “Active Prevalence of Hepatitis C Virus Infection and Molecular Characterization of HCV among Patient in Rwanda”.

With respect to your application for ethical approval to conduct the above stated study at Rwanda Military Hospital, I am pleased to confirm that RMH Ethics Committee has approved your study. This approval lasts for a period of 12 months from the date of this notice, and after which, you will be required to seek another approval if the study is not yet completed.

You are welcome to seek other support or report any other study related matter to the Research Office at Rwanda Military Hospital during the period of approval.

PS: You are required to present the results of your study to RMH Ethics Committee before publication.

Sincerely,

Dr. Pacifique Mugenzi
Co Chair: Rwanda Military Hospital Research Ethics Committee
E-mail: mpaucilfe5@gmail.com

Email: Info@rwandamilitaryhospital.rw
Tel: 0252586120
P.o Box: 3377 RWANDA MILITARY HOSPITAL

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APPENDIX II: Questionnaire administered to Study Participants

Questions

1. Sex


2. How old are you?


3. Where were you born?


4. Where do you stay?


5. Have you travelled outside Rwanda before?


6. What was/is your highest level of education?


7. What work do you do?

8. Have you received blood transfusion before?

9. What is your marital status?

10. How many children do you have?

11. Have you heard of Hepatitis C before?

12. Has anyone in your family died of Hepatitis C before?

13. Is anyone in your family living with Hepatitis C?

14. Have you ever lived with someone with Hepatitis C?

15. Do you know the symptoms of Hepatitis C?

16. If yes, what are they?

17. Have you ever been admitted to the hospital?

18. Have you gone for surgery before?

19. Have you received an organ transplant before?
20. Have been injected with a needle by a traditional doctor / percing /tattoos before?


21. Have you visited a traditional herbal practitioner before?

APPENDIX III: Sequences of 31 HCV RNA positive samples (S1 to S31)
Sequences were obtained from Macrogen Europe Laboratory, Netherlands.