Epidemiology, molecular characterization of hepatitis viruses in Rwanda and implication on liver disease

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To my lovely family

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ABSTRACT

Hepatitis viruses cause major health problems worldwide and, according to WHO, is the seventh leading cause of death globally. It is primarily hepatitis B and C virus that causes chronic hepatitis, liver damage and fibrosis, and long-term risk of cirrhosis and liver cancer. These viruses have different spread globally and shown to cause the majority of deaths in liver diseases, especially in Asia and sub-Saharan Africa. In Rwanda, liver diseases are common and cause 1.5% of annual deaths. As little is known if these liver diseases were caused by viruses or had other etiology, we investigated the presence of serologic markers for HBV, HCV, HDV, HEV and HAV in blood donors and liver disease patients and their matched controls. The persons originated from all five regions of Rwanda and demographic data on the patients and their controls were collected at the same time as they were sampled. The samples were collected between 2014 and 2016. All blood donor samples had been analyzed for HBV and HCV at the National Center for Blood and Transfusion in Kigali, Rwanda.

HBV infection in the form of detectable HBsAg was in samples from 4.1% of the donors, in 3.7% in the controls, and in 31.3% of the liver disease patients. Anti-HBe was more common than HBeAg and was detected in about 70% of HBsAg positive samples. More than 60% of them had detectable HBV DNA. HBV DNA in the samples was sequenced in the structural gene, and family trees were constructed which showed that all HBV strains belonged to subgenotype A1 and 93% of them formed their own branch of the tree, indicating that it is a unique HBV strain spread in Rwanda. No one was shown to have hepatitis delta infection.

For HCV, the picture was different. Primarily, 16% of blood donors' samples had anti-HCV, but 67% of these had false reactivity, so only 5% remained true anti-HCV reactive. Among the controls, 13.4% had anti-HCV compared with 44.3% of the liver disease patients. HCV RNA was detected in 17% of blood donor samples, in 56% of samples from the controls and in 84% of samples from liver disease patients. The virus strains were sequenced and family trees were constructed which showed that the majority of the strains (98.3%) were of genotype 4 and the remainder were genotype 3. The subtypes 4k, 4r, 4q and untyped genotype 4 dominated in samples from all patient groups.

When examining for HEV and HAV markers, 11.7% of all patients had HEV markers. The highest prevalence was found in people from the western and southern provinces where there is a high density of pig breeding. No age-dependent anti-HEV pattern could be identified as opposed to anti-HAV, which increased with age, with fewer anti-HAV positive in persons younger than 25 years compared to the elder group ($p < 0.0001$).

We found in these studies that both HBV and HCV are endemic in Rwanda and caused 74% of the liver diseases, where HCV was more common in patients with liver cirrhosis and liver cancer than HBV. Increased age and female sex were independent risk factors for HCV infection. For HBV, the risk factors were young age, multiple sex partners and male gender. These differences can explain why there were more controls than blood donors that were HCV infected. A risk factor for both HBV and HCV was the presence of people with liver disease in the same household. HEV and HAV were also shown to be endemic, but a decrease in exposure to HAV at younger ages was noted and HEV epidemiology reflected that in countries with possible spread from pigs.

Key words: hepatitis viruses**,** cirrhosis, genotypes, prevalence, Rwanda

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SAMMANFATTNING PÅ SVENSKA

Hepatitvirus orsakar stora hälsoproblem över hela världen och är enligt WHO den sjunde ledande dödsorsaken globalt. Det är främst hepatit B- och C-virus som orsakar kronisk leverinflammation, med leverskada och ärrbildning, och på lång sikt risk för skrumplever (cirrhos) och levercancer. Dessa virus har olika spridning globalt och visats orsaka flertalet dödsfall i leversjukdomar, speciellt i Asien och i Afrika söder om Sahara. I Rwanda är leversjukdomar vanliga och orsakar 1,5% av årliga samtliga dödsfall. Då lite var känt om dessa leversjukdomar var orsakade av virus eller hade annan etiologi, undersökte vi förekomsten av serologiska markörer för HBV, HCV, HDV, HEV samt HAV i prov från blodgivare, och patienter med leversjukdomar och deras matchade kontroller. De undersökta personerna härrörde från samtliga fem regioner i Rwanda och demografiska data på patienterna och deras kontroller samlades in samtidigt som de provtogs. Proverna samlades in mellan 2014 och 2016. Alla blodgivarprov hade analyserats för HBV och HCV på blodcentralen i Kigali i Rwanda.

HBV infektion i form av detekterbart HBsAg fanns i prov från 4.1% av blodgivarna i 3.7% hos kontrollerna, och i 31.3% av de leversjuka patienterna. Anti-HBe var vanligare än HBeAg och påvisades i omkring 70% av de HBsAg positiva proven. Drygt 60% av dem hade påvisbart HBV DNA. Provens HBV DNA sekvenserades i strukturgenen och släktträd byggdes upp som visade att alla HBV-stammar tillhörde subgenotyp Al och 93% av dem bildade en egen gren på trädet, vilket pekar på att det är en unik HBV stam som sprids i Rwanda. Ingen visades ha hepatit delta infektion.

För HCV var bilden annorlunda. Primärt hade 16% av blodgivarna anti-HCV, men 67% av dessa hade falsk reaktivitet, så endast 5% kvarstod som sant anti-HCV reaktiva. Bland kontrollerna hade 13.4% anti-HCV jämfört med 44.3% av de leversjuka patienterna. HCV RNA kunde påvisas i 17 % av proven från blodgivarna, i 56% av proven från kontrollerna och i 84% av proven från de leversjuka patienterna. Virusstammarna sekvenserades och släktträd byggdes upp som visade att majoriteten av HCV-stammarna (98,3%) var av genotyp 4 och de återstående var genotyp 3. Subtyperna 4k, 4r, 4q och otypade genotyp 4 dominerande i prov från alla patientgrupper.

När markörer för HEV och HAV undersöktes, hade i 11,7% av alla HEV-markörer, Den högsta prevalensen fanns hos personer från de västra och södra provinserna där det är en hög densitet av grisuppfödning. Inget åldersberoende mönster för anti-HEV kunde identifieras till skillnad från anti-HAV som ökade

med ökande ålder på personerna, med färre anti-HAV positiva bland individer under 25 år jämfört med de äldre ($p < 0{,}0001$).

Vi fann i dessa studier att både HBV och HCV är endemiska i Rwanda och orsakade 74% av leversjukdomarna, där HCV var vanligare hos patienter med levercirros och levercancer än HBV. Ökad ålder och kvinnlig kön var oberoende riskfaktorer för HCV-infektion. För HBV var riskfaktorerna ung ålder, flera sexpartners och manlig kön. Dessa skillnader kan förklara varför det var fler kontroller än blodgivare som var HCV infekterade. En riskfaktor för både HBV och HCV var förekomsten av personer med leversjukdom i samma hushåll. HEV och HAV visades också vara endemiska, men en minskning av exponering för HAV i yngre åldrar noterades och HEVs epidemiologi återspeglade den hos länder med möjlig smittspridning från svin.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Twagirumugabe T**, Swaibu G, Walker TD, Lindh M, Gahutu JB, Bergström T, Norder H: Hepatitis B virus strains from Rwandan blood donors are genetically similar and form one clade within subgenotype A1. *BMC Infect Dis 2017; 17:32*

II. **Twagirumugabe T**, Swaibu G, Walker TD, Bergström, T Gahutu JB, Norder H: Low prevalence of hepatitis C virus RNA in blood donors with anti-hepatitis C virus reactivity in Rwanda. *Transfusion 2017; 57:2420-2432*

III. **Twagirumugabe T**, Mukabatsinda C, Habarurema S, Seruyange E, Bergström T, Gahutu JB, Walker TD, Norder H: Hepatitis C virus infection is common among females and hepatitis B virus infection among males with liver disease in Rwanda. *Manuscript submitted to BMC Infect Dis*

IV. **Twagirumugabe T**, Saguti F, Habarurema S, Gahutu JB, Bergström T, Norder H: Hepatitis A and E virus infections are common in Rwanda but with different epidemiological patterns. *Manuscript submitted to Intl J Infect Dis*

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ABBREVIATIONS

cccDNA	Covalently closed circular DNA
CMIA	Chemiluminescent microparticle immunoassay
DNA	Desoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HCC	Hepatocellular carcinoma
HDV	Hepatitis *delta* virus
HEV	Hepatitis E virus
HIV	Human immunodeficiency virus
LC	Liver cirrhosis
NA	Nucleoside analogue
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription PCR
RNA	Ribonucleic acid
S/CO	Signal to cut-off
SSA	Sub-Saharan Africa
SVR	Sustainable viral response
WHO	World Health Organization

1. INTRODUCTION

Hepatitis viruses are hepatotropic viruses that mainly cause acute and/or chronic liver inflammation, which may degenerate in some cases into hepatocellular carcinoma and liver cirrhosis. So far, five known hepatitis viruses, A, B, C, *delta* (D) and E can cause liver disease in humans. Of them, hepatitis B and C viruses (HBV and HCV) are leading causes of liver cirrhosis (LC) and hepatocellular carcinoma (HCC) and they are prevalent in more than 60% of patients with liver diseases worldwide[1,2].

Almost 2 billion of the global population has been infected by HBV and more than 240 million have chronic HBV infections, which cause 600,000 to 1 million annual deaths worldwide[3-5]. Chronic HCV infections are prevalent in more than 70 million individuals in the World as well [6,7]. Asian and Sub-Saharan African (SSA) countries are the most affected by both viruses. Prevalence of the chronic HBV infection are estimated to 5-8% in SSA region or even higher in some West-African countries[6,8]. The global prevalence of anti-HCV is 2.3-2.5% and is estimated to 2.9-3.2% in Africa with also geographical differences across the continent[6,9,10].

An effective HBV vaccine exists since 1986 and was recommended by the WHO for all neonates since 1993. Early implementation of this policy in highly endemic South Asian countries has resulted in a decline of HBV incidence[5,11,12]. Despite an overall worldwide decreasing prevalence of chronic HBV infections, the situation did not change and its prevalence is even on raise in some SSA countries [4,8]. In this region, HBV vaccine was been introduced in National Expanded Programs of Immunization as late as in the beginning of 2000s and its impact has not yet been evaluated. Unlike for HBV, there is no vaccine against HCV but the infection can currently be completely cured by new direct

acting antivirals (DAA), which are safer and more effective than previous interferon-based treatments regimen[13].

The epidemiology of other hepatitis viruses in SSA is less well known. Hepatitis *delta* virus (HDV)-HBV coinfection is only reported in Central and Western Africa[14-16]. Enteric hepatitis A (HAV) and E (HEV) viruses are endemic in the region as well and almost all children get HAV infections at young age and acquire a lifelong protective immunity from this infection[17,18]. With improved socioeconomic conditions especially in urban areas, early infections may be infrequent. This may result in an increasing number of individuals without immunity and at risk to HAV infection, which can take a severe course of fulminant hepatitis in old adults[19,20].

HEV is also responsible of acute hepatitis with signs and symptoms similar to those of acute HAV infection. Both are endemic in areas with poor hygiene and limited access to clean water[21]. Episodes of acute HEV or HAV infection are generally self-limiting but acute hepatitis E by genotype 1 has been fatal in more than 20% of infected pregnant women in India and Sudan [22,23]. In addition, HEV infections can take a chronic course in some immunosuppressed patients infected by HEV strains from pigs or acquired from blood transfusion[21,24-28]. Update prevalence of anti-HAV, HEV markers and circulating HEV genotypes remain poorly explored in Africa[17,29].

This thesis focused on epidemiology and molecular characterization of hepatitis viruses in Rwanda, one of SSA countries. It is a small country of 26,338 km^2, located in Eastern African region boarded by Uganda, Tanzania, Democratic Republic of Congo and Burundi in north, east, west and south respectively.

In 2015, the population was estimated to 11.3 million and 53.2% were younger than 20 years[30]. The country is composed of five provinces: Eastern, Western, Southern and Northern Provinces and Kigali city in center. The organization of

the health system is pyramidal and almost parallels that of the administrative system. It starts at community level with community health workers at each village who liaise with health centers. Health centers report and refer patients to district and provincial hospitals. Difficult cases and patients in need of a highly specialized care are referred to national referral hospitals. At national and some provincial hospitals, facilities to diagnose and treat HBV and HCV infections are now being put in place and reinforced to respond to the WHO goal for eradication of HBV and HCV by 2030[31].

Besides this comprehensive geographical access to care, Rwanda has adopted a universal health insurance for all citizens since almost seven years[32]. Several other gaps remain yet to be addressed including data on epidemiology on hepatitis viruses. Small studies have been conducted on specific groups of patients in Kigali prior to 2015. Prevalence for HBsAg and anti-HCV were 5% and 3-5% respectively [33,34]. No study has focused on HEV or anti-HAV and characterization of HBV and HCV strains was performed on only a small number of patients in Kigali city as well[35,36].

1.1 HEPATITIS B VIRUS

1.1.1. Structure, genome and replication

HBV is an enveloped partially double-stranded relaxed-circular DNA virus belonging to *Hepadnaviridae* family. Its genome of about 3.2 kb pairs has four partially overlapping open reading frames (ORF): S or surface, C or core, P or polymerase and X-genes (figure 1). The S gene has three in-frame translation sites as preS1, preS2 and S that have S domain in common. Similarly, C gene has precore and core domains[37]. The different HBV genes encode for 6 proteins vital for viral structure and cycle[38].

The ORFs are encoded in different polyadenylated mRNA transcripts that have different lengths (figure 1)[37,39].The shortest subgenomic mRNA transcript is 0.7 kb long and encodes for the X protein. The second shortest is 2.1 kb and encodes for M (middle) and S (small) surface proteins depending on reading initiation site, at preS2 or S frame respectively. The L (large) surface protein is encoded by a 2.4 kb mRNA transcript whose initiation reading site is at preS1 domain. The longest mRNA transcript has 3.5 kb and encodes for the precore protein. This protein is processed within the endoplasmic reticulum (ER) of the host cell. During this process, first 19aa at N-terminal and 34aa at C-terminal are cleaved off and a final product, the e-antigen (HBeAg), is secreted into plasma and does not take part in the structure of the virion. The core protein and viral polymerase are translated from pregenomic RNA (pgRNA), for which start points are 29-35 bp downstream from that of the precore mRNA. This pgRNA serves also as template for HBV DNA synthesis through reverse transcription[37,39-41]. A core promoter at nucleotide positions 1636 to 1851 of the HBV genome regulates both precore mRNA and pgRNA transcription. The basic core promoter (BCP; at position 1744 to 1851) is required to direct a correct initiation of transcription of those two mRNAs from cccDNA. The

upstream sequence of the core promoter or "core upstream regulatory sequence" (at position 1636 to 1744) activates the transcriptional effect of the BCP[42].

The different mRNAs and pgRNA result from transcription of HBV DNA present in infected hepatocytes. During the infection, HBV binds to hepatocytes via a specific receptor, NTCP (sodium taurocholate co-transporting peptide) and the virus enters by endocytosis into the cytoplasm. From the cytoplasm, the core particle containing partially double stranded HBV DNA releases the HBV DNA into the cellular nucleus where this viral relaxed HBV DNA is completed into a covalently closed circular DNA (cccDNA). The cccDNA remains in the nucleus as a mini-chromosome and serves as template for viral transcription into the different mRNAs and the pgRNA by cellular polymerase[37,38,43-45]. The pgRNA has, at its 5'end, an encapsidation ε signal, a hairpin secondary structure that forms with the viral polymerase a complex involved in packaging and pgRNA encapsidation into newly formed core particles where its reverse transcription into HBV DNA occurs. It is reverse transcribed into an HBV DNA minus strand that will serve as template for synthesis of the plus strand[37,46]. New HBV DNA-containing capsids can get enveloped by surface proteins and lipid bilayer within the ER to form new infective viral particles or the HBV DNA can be reintroduced into the cellular nucleus to replenish the pool of cccDNA[45,47].

The process of HBV replication relies mainly on the viral polymerase, which has different domains playing different roles. The terminal protein (TP) domain binds to 3' end of the pgRNA in the process of encapsidation and allows priming for initiation of the DNA minus strand synthesis. The reverse transcriptase and ribonuclease H domains are involved in the reverse transcription and degradation of the pgRNA during the synthesis of the minus strand HBV DNA respectively [37,46,47].

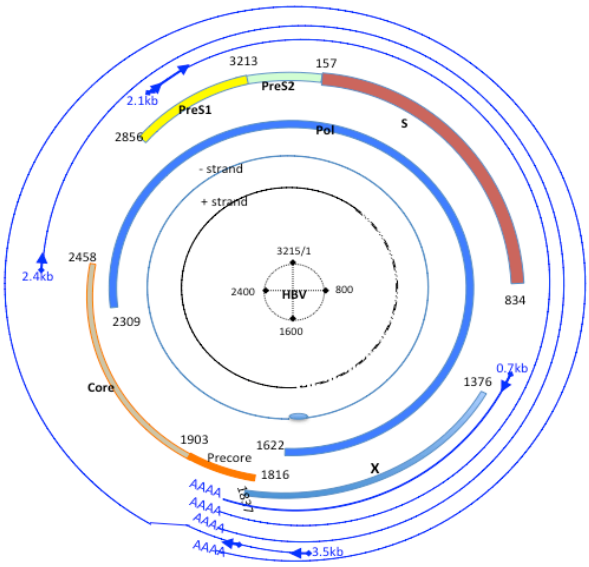


Figure 1. HBV genome represented with the partially double-stranded DNA. The negative strand is complete while the positive one is incomplete. The different genes/ORF and their in-frame translation sites, their starting and ending nucleotide positions are presented. In blue color, the different mRNA transcripts are also represented with their respective lengths in kilobases (kb).

1.1.2. Genetic variability

Genotypes

During the polymerase-dependent HBV replication and transcription, errors in nucleotide incorporation or mutations are frequent since the viral polymerase lacks a proofreading function. Some of the sequence diversities in the genome have been fixed and given rise to different HBV genotypes [37]. HBV genotypes may differ from each other not only by differences in the sequence of nucleotides but also by the length of the genome[37,48]. HBV is classified into 9 different genotypes, A - I, and each of them is further subdivided into subgenotypes designated with Arabic numbers.

Genotypes differ by more than >8% nucleotide sequences of the whole genome whereas subgenotypes are defined by 4-8% nucleotide sequences divergence within a same genotype. To date, subgenotypes are only known for genotypes A, B, C, D and F. Other genotypes do not have sufficient nucleotide sequences heterogeneity to be divided into subgenotypes[48-50]. The genotypes have specific geographical distribution across the globe and may affect differently the progression of the liver disease to severe acute HBV or LC and HCC[50,51].

HBV genotype A strains are mostly isolated in SSA including West-African countries, North Europe, USA, India and in African descents in Haiti and South America[48,51,52]. In Asian countries, a predominance of genotypes B and C but also genotype A are noted[48]. Genotype D is ubiquitous but mainly prevalent in Mediterranean countries, Africa, India and Latin America while genotype E is isolated among individuals from West and Central Africa[48,49,51,53]. Genotype G has been identified in France, Germany, Asia, USA, Mexico and Central America[51,54] while genotypes F and H are respectively found in Mexico, South and Central America[52,54]. HBV genotype I, a genotype A/C recombinant, was found in Southeast Asia[55,56]. A strain of HBV not yet recognized as genotype J close to genotype C and gibbons' genotypes was found from an old Japanese man with HCC in 2009[57].

Mutations and deletions

Mutations can occur along the HBV genome due to polymerase infidelity or immune and therapeutic pressures. Mutants with biological advantage over others and selective advantage over the wild type are naturally selected[58,59]. Mutations of interest are those with proven clinical and pathological effects on the host.

Point mutations at the precore/core ORF can have an impact on secretion of HBeAg, a soluble protein derived from translation of the precore gene that harbors immunomodulatory properties. HBeAg positive status is concomitant with a high replication rate and seroconversion to anti-HBe is generally associated with low levels of HBV DNA in serum. However, HBV strains with some mutations that abolish secretion of HBeAg are associated with high viral load despite anti-HBe positive status. The most common mutation responsible of this abolishment is a change of G to A at nucleotide (nt) 1896 in the encapsidation ε signal of the precore domain[58]. For an efficient HBV replication, a stable and strong ε signal needs a base pairing that stabilizes the hairpin secondary structure. In that regard, the nt 1896, mostly a G in wild type HBV, is paired with nt 1858, which is generally a T except for genotypes A, C1, F2 and F3 which have a C[45,60]. Therefore, the most commonly stable mutation in that region is a G to A switch at nt 1896 creating a pair stabilizing the stem loop of the encapsidation signal. This results in a premature stop codon (TAG) and synthesis of the precore protein is limited at its N-terminal 28th residue. This mutation is mainly found in HBV genotypes B, C, D and E for the reason mentioned above [37,61].

Beside this common translational mutation in precore region, other mutations in the core promoter region, and particularly in the BCP, affect the transcription of the precore mRNA without seriously affecting that of pgRNA. Such mutations reduce expression of HBeAg without affecting HBV DNA replication. The most common mutation in the BCP is a double mutation, A1762T/G1764A[41,60]. Infection by HBV strains with reduced expression of HBeAg clinically predisposes to acute fulminant hepatitis and to a higher progression to LC and HCC[62-64].

Mutations can also occur at the level of the preS/S gene, which encodes for the envelope proteins or HBsAg. The surface protein harbors from its preS1-

encoded region a site expressed at luminal side of virion surface, the NTCP, which serves for attachment to the specific receptor on hepatocytes[65].

Also, surface proteins are immunogenic and target of neutralizing anti-HBs. Epitopes recognized by neutralizing cytotoxic T-cell and B-cell are expressed at the C-terminal part of the pre-S1 and the N-terminal part of the pre-S2 region and most importantly at the S region[66]. Changes in the amino acids 122, 127 and 160 in the S protein have been used to define subtypes. A substitution glycine-arginine at amino acid position 145 has been linked with development of active HBV infection in vaccinated individuals (immune escape status) [59,67,68]. This mutation is the most common escape mutation in the S-region but others like Pro120Thr, Gln129His, Gly130Met, Met133Leu, Ser143Leu and Asp144Ala have also been described[69-71].

The most selective mutations observed in the pre-S1 and pre-S2 regions are deletions of a number of nucleotides that can involve both the C-terminal part of the preS1 and preS2 parts or of the preS2 alone. These deletions may influence pathogenesis and outcomes of an HBV infection including prediction of the progression to HCC. Large surface proteins with partial deletions of pre-S2 have had their secretion impaired with a consecutive accumulation in the endoplasmic reticulum. This accumulation leads to oxidative stress on the hepatocyte and may result in cellular DNA damage and genomic instability, and "ground glass hepatocytes" type II formation, which may potentially lead to progression to LC and HCC [72-75].

Therapeutic agents for chronic hepatitis B infection, mainly nucleoside analogues polymerase inhibitors especially lamivudine, have been associated with generation of resistant mutants. Since the P-gene, with particularly the reverse transcriptase domain, fully overlaps the S-gene, some mutations in the

P-gene may also cause mutations in the S-gene region with potential immune escape status and diagnostic challenges for HBsAg-based assays [71,76-80].

Finally, mutations can also occur in the X-gene. This gene partially overlaps the core promoter region. Thus, mutations in that region like A1762T/G1764A interfering with HBeAg secretion may also impact on the X-gene[58].

1.1.3. Clinical presentation of HBV infection and diagnosis

Mode of transmission

HBV is a blood-borne virus transmitted between humans via blood exchange or mucosal contact with body fluids such as blood products, semen and saliva[81]. The majority of HBV infected individuals in highly endemic countries of Asia have acquired the infection by vertical transmission route during birth. Infections acquired during birth lead to chronic hepatitis B in more than 90% compared to 25-50% and 6-10% when infections are acquired via horizontal transmission at 1-5 years of age and in childhood or adulthood respectively [81].

The horizontal transmission predominates in the rest of the World and may occur through unprotected sexual intercourses with HBV-infected persons, contacts with infected body fluids via skin cut, scratches or other traumatic injuries possible at different occasions in daily life. IV drug abusers get infected from shared syringes with infected individuals[82,83]. Of note, HBV may remain stable for up to 7 days at room temperature and 8 months in needles[81,84].

Acute hepatitis B

About 1-4 months after the inoculation of HBV, infected individuals may have clinically apparent symptoms of acute hepatitis. However, acute hepatitis B is

asymptomatic in more than 2/3 of the cases. The pathogenesis of HBV infection is immune-mediated. Due to immature cell-mediated immunity in neonates and young children, symptomatic acute hepatitis is most likely to happen in adults[43]. Signs and symptoms are not specific to hepatitis B and may include nausea, fatigue, headaches, jaundice and dark urine preceded by mild fever and arthralgia[85]. Hepatomegaly and splenomegaly can be associated. In less than 5% of the cases with symptomatic acute hepatitis B, fulminant hepatic failure may occur. It is characterized by development liver encephalopathy within 8 weeks of onset of jaundice and important coagulation disorders. This can lead to death and need for life-saving liver transplantation[86,87].

All signs and symptoms are concomitant with elevated alanine aminotransferase (ALT) to more than 10 times the normal values and elevated bilirubin. Detection of HBsAg with anti-HBc immunoglobulin (Ig) M and with or without HBeAg, and HBV DNA are needed to make the diagnosis of acute hepatitis B[88].

Chronic hepatitis B

Signs and symptoms of overt acute hepatitis B last about 1-3 months except fatigue that may persist. This phase may be followed by a clearance of HBV infection materialized by HBV DNA clearance and HBsAg seroconversion to anti-HBs. In case of persistence of HBsAg for more than 6 months after its first detection, hepatitis B has followed a chronic course[89]. There is no positive correlation between severity of acute hepatitis B and the rate of progression to chronic hepatitis[86,90].

Chronic hepatitis B is generally asymptomatic except during flares of HBV reactivation involving host cell-mediated immune system that may be associated with minor symptoms like low-grade fever, fatigue, headaches and elevated ALT concomitant with loss of HBeAg positive status[91]. Chronic hepatitis B infections often have similar symptoms and laboratory findings as those of acute hepatitis

B. However, anti-HBc IgM titers in the chronic phase are lower or absent, and the HBV DNA load may be higher than in the acute hepatitis B infection[86,92,93].

Chronic hepatitis B is divided into different unpredictable and non-consecutive so-called "phases" based on interaction between the virus and the immune response of the host. They are clinically determined based on HBeAg status, viral load, ALT levels, presence and importance of liver fibrosis (table 1)[85,89].

Table 1. Different phases of chronic HBV infection

Phase	*HBeAg*	*Anti-HBe*	*HBV DNA level*	*ALT levels*	*Histology of the liver*	*Comments*
Chronic HBV infection	Positive	Negative	High; $>10^7$	Normal	No inflammation	Immune tolerance; Young patients <30years
Chronic hepatitis B	Positive	Negative	High; $>10^4$	Elevated	Inflammation present	Immune reactive; Flares, ends with loss of HBeAg
Chronic HBV infection	Negative	Positive	Low; $<10^3$	Normal	None or Minimal	Inactive carrier
Chronic hepatitis B	Negative	Positive	Low to High	Elevated	Inflammation or Fibrosis	Immune escape; PC/BCP mutants

ALT= alanine aminotransferase; PC=pre-core; BCP: basal core promoter; HBV DNA in IU/mL

The immune tolerance phase is relatively longer in children who have acquired the infection perinatally than in adults who contracted the infection in the late childhood or adulthood[94-96]. In few cases, viraemia can coexist with loss of HBsAg. This status known as "occult hepatitis B infection" and is characterized by lack of HBsAg reactivity, presence of total anti-HBc with or without anti-HBs. Elevated liver enzymes are observed with low viraemia and cccDNA in

hepatocytes raising a permanent risk of reactivation in case of immune suppression[89,97]. However, most individuals who have cleared the HBV infection harbor cccDNA within the hepatocytes without having active infection[98].

Liver cirrhosis (LC)

One of worst outcomes of chronic hepatitis B is progression to LC, which occurs in 15-40% of the patients with chronic hepatitis B all life long[85]. Factors for this progression are a high viral load >2000IU/ml, male gender and old age with persistent infection. HBeAg positive status, frequent flares or elevated ALT, BCP mutants and infection by HBV genotype C versus B or D predispose to a higher risk of LC as well[85,96,99,100].

LC is clinically characterized by advanced liver fibrosis with conserved liver functions or associated with low albumin and low platelets. Coagulation disorders, portal hypertension, ascites and possibly liver encephalopathy can be found when the cirrhosis is decompensated. A persistently high viral load is among the leading factors to decompensation of the cirrhosis and a factor to low survival rate of patients with cirrhosis[101]. Although HCC may occur in patients with or without LC, a high viral load is a risk factor to the development of HCC in cirrhotic patients[102-104].

Hepatocellular carcinoma (HCC)

HCC following chronic HBV infection has been reported in up to 5% of the cases. However, progression to HCC can reach 15% after 13 years of follow up in CHB diagnosed with HBV DNA levels higher than 200,000 IU/ml. Other factors predisposing to HCC are smoking, intoxication with aflatoxin, alcohol abuse, male gender and advanced age with HBV infection [100,102]. Also, diabetes mellitus, co-infection with HCV, HDV, HIV and HBV genotype C, mutations

in the pre-C/C and/or deletions in the pre-S gene are also among factors to HCC in chronically HBV-infected patients[105,106]. In SSA region, HBV-induced HCC tends to occur in much younger patients than in other regions in the world. There is a high predominance of subgenotype A1 in SSA but the mechanisms by which subgenotype A1 affects the development of HCC remains unknown and needs to be investigated[107,108].

The diagnosis of HCC is based on abdominal ultrasound with identification of a solid liver tumor [109]. Alpha-fetoprotein levels can be useful in detecting early stages of HCC but have a sensitivity of as low as 66%[110,111]. Strong positive diagnosis is therefore based on histology[112]. However, HCC is asymptomatic in its early stages and the majority of the cases are diagnosed at advanced stages when resectability of the tumor is impossible especially in resource-limited settings. Moreover, delayed diagnosis is most common when HCC occurs in absence of liver cirrhosis [65,113,114].

1.1.4. HBV and HDV dual Infection

HDV is a small negative sense single-strand RNA virus. Its genome of about 1,679 nt has a single ORF encoding for two delta antigens, small (S-HDAg) that supports the replication in the nucleus and the large antigen (L-HDAg) needed for viral assembly. The virus uses cellular RNA polymerase for a rolling circle replication. The viral RNA encodes for a ribozyme, which cleaves and circulates the synthetized RNA into unit length HDV RNA. Both S-HDAg and L-HDAg participate in this replication with S-HDAg being involved in its initiation while L-HDAg participates in the replication modulation and assembly of new virions[115,116]. These virions are enveloped by HBV surface proteins and use these proteins to attach to the specific receptors, NTCP, to infect the liver cells[117]. Superinfection of HBV is therefore obligatory. Simultaneous HBV-

HDV infections lead to spontaneous resolution of the hepatitis in 95% of the cases while the HDV superinfection of chronic HBV causes severe acute chronic hepatitis and tends to lead to a higher rate of progression to LC and HCC than HBV monoinfection[106,116,118-121].

There are 15-20 millions of HBV/HDV-infected individuals worldwide and in SSA region, Central and Western parts are among the most affected[122,15,106].

1.1.5. Diagnostic approach to HBV-induced hepatitis

The diagnosis of active HBV infection is made upon detection of HBsAg. This antigen is the first marker to appear in plasma and remains present during the active infection. Clearance of HBsAg and seroconversion to anti-HBs is generally a sign of recovery from infection while its persistence beyond 6 months after initial diagnosis is a marker of chronic HBV infection [88]. Other serological markers may coexist with HBsAg or anti-HBs. HBeAg is an indicator of an active high replicative state and infectivity as it is associated with high levels of HBV DNA. Seroconversion to anti-HBe generally coincides with clearance of HBV DNA except for infection by strains harboring the mutations abolishing the secretion of HBeAg in which anti-HBe positive status and high viral load may coexist[41]. During an active HBV infection, core antigens (HBcAg) are abundant in the hepatocytes and anti-HBc antibodies almost appear in plasma at the same time as HBsAg[123]. Anti-HBc IgM is present during the acute hepatitis while anti-HBc IgG remains positive in both active and cured HBV infection. Interpretations for different markers are summarized in table 2.

HBV DNA levels are obtained by quantitative real-time PCR from amplification of highly conserved regions within the HBV genome by specific primers and probes[124]. The HBV DNA level is generally useful for the diagnosis of an active

infection and is essential for decision on initiation of treatment, follow-up and in prediction of risk to LC and HCC[102,125].

PCR amplification for whole genome sequencing and phylogenetic analysis are necessary for identification of a new HBV genotype or subgenotype. Partial sequencing of the S-gene is however reliable for already known genotypes and subtypes[126]. Mutations are identified by using PCR amplifications and sequencing of corresponding genes or regions [48,127].

Table 2. Interpretations of HBV serological markers

	HBsAg	*Anti-HBc*	*Anti-HBc IgM*	*Anti-HBs*
Acute HBV infection	+	+	+	-
Chronic HBV infection	+	+	+/-	-
Resolved infection with acquired immunity	-	+	-	+
Occult HBV infection*	-	+	-	+/-
Immunity from the HBV vaccine	-	-	-	+

*HBV DNA is detected generally in low viral load, which differentiates this status from a resolved infection

1.1.6. Treatment of HBV infection

Treatment of HBV is generally indicated for chronic HBV infection. The aims are to clear the virus, to restore the liver function to normal and to reduce the progression to advanced forms of liver disease[95,128]. Nucleoside analogs (NA) that inhibit viral replication by interfering with reverse transcription of the minus strand DNA from pgRNA are currently the cornerstone for HBV treatment[128-131].

Indications for treatment initiation include a persistently elevated HBV DNA with signs of hepatocytolysis. Patients with a viral load >2,000 IU/ml with

moderate inflammation or fibrosis at liver biopsy are candidate to the initiation of anti-HBV therapy. However, indications are also extended to patients with chronic hepatitis with HBV DNA levels higher than 20,000 IU/ml without any need of liver biopsy[89,95]. Although NAs are effective in suppressing HBV replication, they cannot completely eradicate the infection since they do not have effects on the cccDNA[76,132].

To boost the immune system by conventional and pegylated interferon alpha is an alternative to NA[133]. Treatment with interferon has been associated with a reduced rate of progression to severe liver disease [128,134,135]. However, the efficacy in clearing HBV DNA does not exceed 30%[133,136]. Interferon-based course of treatment is generally limited to 48 weeks[89].

1.1.7. Prevention of HBV infection

Vaccination

HBV infections are preventable by an effective vaccine. The HBV vaccine is particularly indicated for newborns and persons at risk such as healthcare professionals, support staff in health facilities, IV drug abusers or individuals living with infected persons[137]. The vaccine is based on inactivated HBsAg obtained by DNA recombination. WHO has recommended vaccination of all children since 1993 and the vaccine is generally provided in 3-doses combined with diphtheria-tetanus-pertussis vaccines[138,139]. A monovalent HBV vaccine is also available[138].

Prevention of mother to child transmission of HBV

In highly endemic areas, neonates from HBV childbearing mothers are at high risk to get infected at birth especially if mothers have HBeAg and consequently a high HBV DNA common in persons infected by genotypes B and C[140,141].

NAs administered to infected mothers during the third trimester combined with immediate initiation of postnatal active vaccination and immunotherapy is effective in reducing the vertical transmission[142,143]. Immunoglobulins and the first dose of the vaccine and are administered after birth within 24 hours, and remaining doses of the vaccine within the regular neonatal and infant immunization calendar[89,143].

1.2 HEPATITIS C VIRUS

1.2.1. HCV genome structure

HCV is a positive single-stranded RNA virus in the *Hepacivirus* genus within the *Flaviviridae* family[144]. Its genome is about 9,6 kb long with a single ORF encoding a polyprotein. The genome translation starts with recruitment and binding of 40S ribosomal subunits to an internal ribosome entry site (IRES) located at its 5'-untranslated region[145]. This translation results in the single 3,000-aminoacids polyprotein, which is cleaved by cellular and viral proteases into three structural proteins forming the viral particles and seven non-structural proteins[144]. The structural proteins are the core forming nucleocapsid, E1 and E2 envelope glycoproteins that play a role in viral host cell entry and are anchored in the lipid bilayer of the viral envelope[146,147]. The non-structural proteins include the p7 or viroporin, which is a membrane Ca ion channel, the NS2 protein that forms a protease with 1/3 of the N-terminal part of the NS3 protein, and cleaves the NS2-NS3 junction. The rest of the NS3 protein forms with its cofactor NS4A a complex NS3/NS4A with protease and helicase/NTPase activities. This complex cleaves the remaining part of the polyprotein into its active proteins: NS3, NS4A, NS4B, NS5A and NS5B. The NS4B induces membranous web formations in the cytoplasm of the infected hepatocyte in which the HCV replication occur protected from cellular nucleases. The NS5A protein modulates interferon response and is involved in viral replication via modulatory effects on NS5B, which is a viral RNA-dependent RNA polymerase (figure 2) [144,145,148].

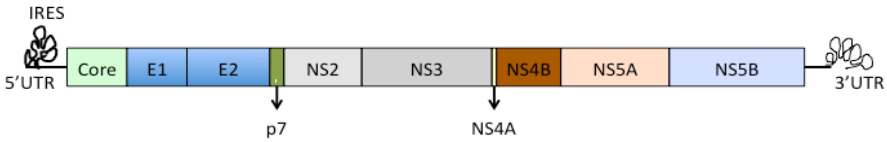


Figure 2: Hepatitis C virus genome in its single ORF flanked at 5' and 3'ends by untranslated regions (UTR). The components of the polyprotein are represented: Core, glycoprotein E1 and E2 for structural proteins, p7 to NS5B for non-structural proteins. The Internal Ribosome Entry Site (IRES) that mediates the translation is also shown within the 5'UTR.

1.2.2. Genotypes

The HCV RNA dependent RNA polymerase encoded by NS5B lacks proofreading functions. Divergent strains and mutants including different genotypes and subtypes have arisen from this error-prone replication. Genotypes are distinguished by more than 30% nucleotide divergence between complete genomes while 15-30% difference within the same genotype is required to define different HCV subtypes [149,150]. There are seven HCV genotypes, 1-7, and more than 67 alphabetically named subtypes[149-152].

HCV genotypes 1 and 3 are ubiquitous while genotype 4 is represented by the majority of strains isolated from Africa including Egypt, a country that historically has the highest prevalence in the World. Genotype 5 is also reported from Africa but mostly in Southern African region[6,151]. A high prevalence of genotype 2, and to the lesser extent genotypes 1 and 3, is reported from the West African region [6,153]. HCV genotype 6 is mostly isolated in South East Asia[151] and genotype 7 has only been recently described in 4 patients from Democratic Republic of Congo[149,152].

Inter-genotypic recombinants can naturally occur and recombinants 2k/1b, initially isolated in Russia, have also been reported from other European countries[154,155]. Other recombinants as 2b/2a, 2/5, 2/6 and 4/1 have been

identified in USA, France, Vietnam and Cameroon respectively and inter-subtypes recombinants are also reported[156-159].

The clinical importance of genotypes initially resided in prediction of response to the interferon-based treatment regimen. HCV genotypes 1 and 4 were the most difficult to treat, as achievement of a sustainable viral response (SVR) was possible for less than 50% of cases compared to more than 75% for those infected with HCV genotypes 2 or 3 despite a longer course of treatment[160]. With DAAs, which have a higher antiviral response, the importance of genotyping remains also crucial for determination of appropriate regimen until an efficient pan-genotypic treatment will be found. Currently, genotype 3 has become the most difficult to treat with these DAAs[161-164].

The role of HCV genotypes in severity of liver disease is not elucidated[165]. HCV genotype 3 has been associated with severe forms of liver disease among IV drug abusers in France and genotype 1b linked to a higher rate of HCC among cirrhotic patients[166]. Likewise, in a recent study conducted in Southeast Asia, Lee et al. have found that genotype 6 was more likely to cause HCC than other genotypes[167,168]. All these studies have been conducted in different settings with divergent prevalence of HCV genotypes and genetic difference between populations, which makes objective comparisons of genotype-specific liver disease severity difficult[169,170].

1.2.3. Clinical presentation of HCV-induced liver disease

Mode of transmission

HCV is a blood-borne virus and several individuals have been infected via unscreened blood products before systematic HCV screening of products before transfusion were implemented in 1990[171]. Sharing needles between IV drug abusers or sharing other sharp objects with infected individuals are

nowadays major routes for spreading the virus[172]. The main routes of HCV infections in resource-limited settings are probably ritual practices among indigenous populations, and also nosocomial infections through unsafe invasive procedures with reuse of contaminated or sub-optimally sterilized instruments [173,174]. Sexual transmission is lower compared to HBV but may be increased in HIV-infected men having sex with men (MSM)[175]. Vertical transmission is less also frequent than that of HBV but may increase if the mothers are HIV-coinfected[176].

Acute HCV infection

One to two weeks after exposure to a high infecting dose of HCV as by blood transfusion, symptoms of acute infection may occur with a high viral load with or without detectable anti-HCV antibodies. However, signs and symptoms are present in less than 20% of the cases. They are flu-like symptoms, abdominal pain, anorexia, icterus and dark urine with increased level of transaminases to more than 10-fold the upper limit of normal range and hyperbilirubinemia[177]. Acute fulminant hepatitis C is rare but more frequent in case of preexisting active HBV infection and vice versa[86,178]. This pauci-symptomatic pattern of acute HCV infection hinders its early diagnosis [177,179]. Therefore, to make a diagnosis, exclusion of another potential cause of acute hepatitis is paramount as there is no specific marker for acute HCV infection unless lack of markers for HCV was identified in samples drawn within the last 6 months prior to the current diagnosis[161]. Following the acute phase, spontaneous clearance of HCV RNA within 6 months occurs in 15-40% while the remaining cases will progress to chronic infection[170,180].

Chronic HCV infection

During the acute phase, seroconversion to anti-HCV occurs early and anti-HCV coexist with detectable HCV RNA[161]. In this phase, mildly elevated or normal

liver enzymes and histological damage of the liver such as necroinflammatory process and liver fibrosis can be observed. Other non-specific biological findings like a thrombocytopenia may also be observed[181]. The rate of spontaneous viral clearance in this phase is rare and the majority of the individuals will remain viraemic. Factors for progression to chronicity are old age at time of the infection, male gender, alcohol abuse and host genetic factors like polymorphism at interleukin 28B gene[169,170,182,183].

The development of advanced life-threatening forms of chronic liver disease is generally slow. Individuals with chronic HCV infection are at risk of up to 20% to progress to LC within 20 years with the infection[184].

HCV-induced liver cirrhosis (LC)

Permanent HCV-induced cytolysis may lead to liver fibrosis and formation of regenerative nodules surrounded by fibrotic septa that characterizes the LC[185]. Chronic hepatitis C evolves towards LC in about 20-30% of cases over a period of 20-30 years[177,184]. Alcohol abuse, older age with HCV infection, coinfection with HIV and diabetes are major contributors to this progression[186,187].

Clinical presentation of HCV-induced LC is not specific and is generally indolent or subclinical until it reaches advanced stages or decompensation. Treatment initiated at the stage of LC has been associated with attenuated progression of the fibrosis and reduction of development of liver malignancy in patients achieving an SVR including patients with already decompensated cirrhosis[188-190].

Furthermore, in patients achieving the SVR, mortality related to liver disease is decreased but also that related to comorbidities at a comparable rate as matched general population thanks to curative effects on HCV extrahepatic disorders [188,191,192].

Individuals with HCV-related cirrhosis carry a permanent annual risk of 1-7% of developing an HCC. HCC is the second leading cause of cancer-related death worldwide and HCV is the most important cause in the World except in Sub-Saharan African countries and in Asia where HBV is highly endemic[113,193,194].

In HCV-infected persons, HCC tends to be more associated with LC as a result of indirect oncogenic effect of HCV via a persistent inflammatory process, liver cell death and proliferation. HCC affects more females and old patients than HBV worldwide[2,113]. The age difference in HCV-induced HCC compared to HBV is even much more important in SSA region[195].

Although the DAAs can cure HCV-related LC, cirrhotic patients with cured infection continue to carry 1% yearly risk of developing HCC especially when factors like diabetes, metabolic syndrome or advanced age are associated [191,196-199]. Such patients should therefore remain candidate to a regular follow-up as those with untreated chronic HCV infection or LC[112].

1.2.4. Diagnosis of HCV infection

The diagnosis for HCV infection is based on detection of anti-HCV antibodies except in case of a recent massive exposure (<4 weeks) with presence of symptoms as 30-50% of cases may lack the antibodies early in the infection and they may also be absent in immunosuppressed patients[161,200]. The anti-HCV assays use enzyme-linked immunoassays (ELISA) techniques. Anti-HCV IgM is present in up to 94% of acute HCV infection but also in more than 50% in persons with chronic HCV. Hence, this assay is unreliable for diagnosing acute HCV infection[201]. The presence of anti-HCV does not necessarily link to an ongoing infection, since the antibodies remain after clearance of the infection. Ongoing HCV is therefore rather proven by detection of HCV RNA by

quantitative PCR. Moreover, in some cases, there is a high rate of false positive results for anti-HCV by ELISA, especially in developing countries[202,203]. Therefore, anti-HCV reactive samples with no detectable HCV RNA should be subject to a confirmatory line immunoblot assay to confirm a cured infection or possible ongoing infection with low level of viraemia [204].

During replicative phases of HCV, nucleocapsid peptides or core antigen (HCVcAg) are released into the plasma and their detection may be used as an alternative to HCV RNA for the diagnosis of active HCV infection[205]. These peptides are highly antigenic and their detection have shown a good correlation with HCV RNA levels except for viral loads lower than 3,000 IU/ml [206-209].The table 3 summarizes the interpretation of HCV markers.

Table 3. Diagnostic approach of HCV infection

Anti-HCV (ELISA)	*HCV core Ag*	*HCV RNA*	*Line Immunoblot assay*	*Interpretation*
Negative	Positive	Positive	Not recommended	Recent HCV infection or infection in patients with immune deficiency
Negative	Negative	Positive	Not recommended	Idem as above but with low viraemia
Negative	Negative	Negative	Not recommended	No current or past HCV infection
Positive	Positive or negative	Positive	Not recommended	Acute HCV infection if exposure within last 6 months, chronic HCV infection if more than 6 months
Positive	Negative	Negative	Positive	Resolved HCV infection or low level of HCV viraemia
Positive	Negative	Negative	Negative	False positive anti-HCV antibodies

Molecular characterization or genotyping of HCV strains is crucial for assignment of treatments regimen to infected patients. Genotyping is performed by whole genome sequencing for identification of new genotypes or recombinants. Sequencing on limited regions of NS5B or core is acceptable for

identification of known genotypes. However, sequencing the core region does not allow identification of subtypes as efficiently as the NS5B region[210,211].

1.2.5. Treatment of HCV infection

About 15-40% of HCV infected patients may spontaneously clear the infection during the first 6 months of the infection[182]. The remaining patients are at risk to develop chronic hepatitis and are therefore candidate to treatment in order to prevent progression to LC and hepatocellular carcinoma. However, HCV has a slow progression to those life-threatening conditions and in resources-limited settings, priority can be given to cases with advanced fibrosis[212]. Nevertheless, the risk to develop HCC is 1% per year for patients with LC achieving a SVR, and this plays in favor of an earlier initiation of the treatment[199].

Effective treatment for HCV infection relied prior to 2011 on interferon-based regimen comprising interferon α, or polyethylene glycol (pegylated)-interferon for its more prolonged half-life and activity, and ribavirin. However, in these regimen, sustainable viral responses (SVR) could be achieved in up to 80% of cases infected by HCV genotypes 2, 3, 5 and 6 and in only less than 52% for HCV genotypes 1 or 4-infected persons[213,214]. Many patients could not comply with this 24-48 weeks course of treatment mainly because of interferon-induced side effects such as headaches, fatigue and depression and side effects of ribavirin like nausea and anemia[215].

Currently, DAA comprising different protease and enzymatic inhibitors targeting the main proteins involved in life cycle of HCV have been introduced and superseded the interferon-based regimen. They have shown improved tolerability and efficacy with a shortened course of treatment[216]. Different DAA exerting effects at different sites are available. They are NS3/4A protease, NS5A or NS5B inhibitors. For NS3/NS4A the protease inhibitors used are telaprevir, boceprevir, simeprevir, grazoprevir, paritaprevir, asunaprevir, voxiprevir and

glecaprevir. Daclatasvir, ledipasvir, elbasvir, ombutasvir, velpatasvir, pibrentasvir and odalasvir are NS5A inhibitors while sofosbuvir and dasabuvir interfere with NS5B[163,216]. For current treatment guidelines, combinations of two or three DAA with or without ribavirin are used. Different regimens are assigned according to HCV genotypes and antiviral response may differ between different genotypes as well[161,217,218]. Although SVRs following DAA remain >90%, success of the treatment may be hindered by naturally occurring and drug induced resistant mutants[218,219].

1.3 HEPATITIS E VIRUS

Hepatitis E was first described in 1978 in Kashmir-India during an epidemic defined as of non-A non-B acute hepatitis involving almost 52,000 persons[220,221]. The virus was later discovered by Balayan et al. in 1983[222]. HEV has been initially thought exclusively transmitted via fecal-oral route and highly endemic in developing nations with poor hygiene and lack of access to clean water. It is nowadays recognized as an important public health problem also in the developed world[223]. Several animals are reservoir of the virus, especially swine and wild boar, and can transmit it to humans via direct contact or contact with their fecal products but the predominant route is probably via consumption of undercooked meat or meat products from infected animals[220,224].

1.3.1. Structure of the HEV genome

HEV is a small single-stranded non-enveloped RNA virus belonging to *Hepeviridae* family. HEV strains infecting mammalians are classified into the *Orthohepevirus* A genus. HEV genome is about 7.2 kb long and encodes for three ORFs with ORF1 being the largest and coding for nonstructural proteins involved in viral replication and protein processing. Proteins involved in replication are methyltransferase (MeT), the helicase (Hel) and the RNA dependent RNA polymerase (RdRp).

The papain-like cysteine protease (PCP) is another non-structural protein that may be responsible of processing the polyprotein derived from ORF1 into functional proteins. Other proteins encoded by ORF1 are Y and X domains whose functions are unknown and a highly variable domain (HVR) near the PCP [225,226]. ORF2 encodes for the capsid[227]. ORF3 partially overlaps the ORF2 and may encode for a small protein involved in secretion of the virions[226-229]. A junction region is located between the stop codon for ORF1 and reading

initiation codon for ORF2/3 and encodes a stem-loop RNA secondary structure involved in viral replication (figure 3)[226].

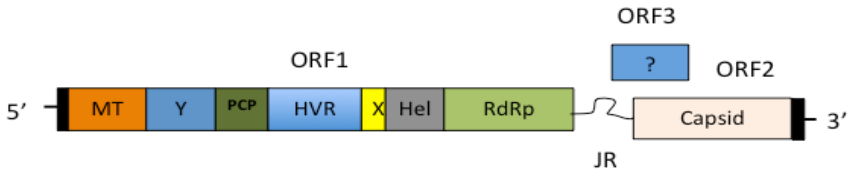


Figure 3: Genomic organization of the HEV showing the 3 overlapping open reading frames:ORF1 encoding for non-structural proteins, ORF2 for the capsid and ORF3 whose role remains elusive. (Different domains of ORF1 here represented: MT: methyl-transferase; Y: Y-domain; PCP: papain-like cysteine protease: X domain; HVR: Highly Variable domain; Hel: helicase; RdRp: RNA dependent RNA polymerase; JR: Junction region)

1.3.2. HEV genotypes and global distribution of HEV infecting humans

The *Orthohepevirus A* genus encompasses eight different genotypes (HEV1-HEV8) based on the sequence of whole genome with over 19% nucleotide divergence [225,230]. HEV1 and HEV2 are obligatory human pathogens and mainly transmitted via fecal-oral route. HEV3 infects both humans and animals such as pigs, deer, rabbits, goats and mongooses[224,231,232]. HEV4 circulates among swine and humans but has been also identified in cow milk in China[28,225,233]. HEV5 and HEV6 have been isolated from wild boars in Japan while HEV7 and HEV8 were found in camelids[225]. Recently, HEV7 has caused a chronic liver disease in liver transplanted patient from United Arab Emirates[234,235]. One HEV strain infecting moose has been identified, although it is not known if the virus can infect humans[236].

All these genotypes are differently distributed across the globe. HEV1 has been reported in different outbreaks and sporadic cases of acute hepatitis in Africa and Asia[29]. HEV2 was found in Mexico and West, Central and Southern African

countries like Nigeria, Chad and Namibia[237]. HEV3 has mainly been isolated in the USA, Western Europe, New Zealand, Japan and Korea while HEV4 was described from China, Taiwan, Japan and Vietnam but also from some cases in Europe[237,238].

1.3.3. Clinical presentation of HEV infection

Mode of transmission

HEV is mainly transmitted by fecal-oral route usually from drinking unclean water contaminated by infected individuals or animals. HEV outbreaks have been reported in Asia and Africa, mostly occurring in refugee camps or zones where drinking water is contaminated by sewage[239-242]. This water-borne transmission is relatively common for HEV1 and HEV2 and a human-to-human transmission remains rare but possible[243]. In developed countries, transmission via a foodborne route is the most predominant through consumption of raw or undercooked meat and other products from infected pigs [244-246]. It has been shown that a complete inactivation of HEV is only obtained by heating at >71° degrees Celsius for at least 20 minutes[244].

However, individuals can also get infected via close contact with infected animals, or animal products [223,245,247]. Transfusion-transmitted HEV infections by blood products have also been repeatedly reported in developed countries[24,248,249]. Finally, mother-to-child transmission remains not thoroughly explored but it has been raised that it may occur in more than 50% of babies born from viraemic mothers infected by HEV1[250-252].

Acute HEV infection

After an incubation period of 15-60 days, a symptomatic acute hepatitis caused by HEV1 or HEV2 occurs in less than 20% of cases and remaining cases will

feature a subclinical course [253]. Symptoms do not differ from that of acute hepatitis caused by hepatitis A virus (HAV), another RNA virus from the *Picornaviridae* family[254,255]. Signs and symptoms, when they are present, include fatigue, mild fever, malaise, jaundice, anorexia, vomiting, hepatomegaly and pruritus[255,256]. They are concomitant with increased liver enzymes and hyperbilirubinemia. In more than 98% of cases, the symptoms will resolve spontaneously without treatment within a period of 4-6 weeks but in HEV1-infected pregnant women especially during the third trimester, progression to liver failure is relatively more common with a case-fatality rate that can be as high as 30% or beyond [23,29,257,258]. Inhibition of estrogen receptors by HEV infection in pregnant women and delayed interferon β response resulting in an increased viral replication is putative mechanism to this progression[259-261]. Immunosuppressive therapy can also be associated with worse prognosis[261,262].

Acute manifestations for HEV3 infections were described in sporadic cases from developed nations with a severe clinical course in pregnant women as well but no death has been deplored so far. The difference in virulence or in health facilities settings could explain the different prognosis between HEV1 and HEV3 but cases of HEV3 infection in pregnant women remains sporadic[263-266].

Acute hepatitis E in patients with preexisting chronic hepatitis also features a severe clinical pattern as it can cause a decompensation to liver failure with apparent clinical jaundice, coagulation disorders and is associated with a poor prognosis in up to 2/3 cases[25,267-269]. HEV1 and HEV2 infections are also associated with a higher severity and poorer prognosis than HAV infection in acute on chronic liver failure[270].

Chronic hepatitis E

Once infected by HEV, some patients with immune deficiency are unable to clear the virus, which may then persist, replicate and cause chronic hepatitis[271].

First cases of HEV persistence for more than 6 months were described by 2008 in France from solid-organs recipients [272]. Several similar cases among solid-organs recipients, patients with hematological cancers undergoing chemotherapy [273,274] and HIV-infected patients with low CD4 count have been reported as well[275-277]. Chronic hepatitis E generally shows a cryptogenic clinical pattern with mild to moderate signs or symptoms limited to fatigue, moderately elevated liver enzymes and jaundice[278]. Cases of LC including those with rapid progression within less than 3 years from initial HEV infection have been reported in HIV-infected patients[279,280]. The majority of these cases involved HEV3 infection. Limited studies in SSA where both HIV and HEV1 or HEV2 are highly endemic did not show any case of HEV-induced chronic hepatitis despite a relatively important proportion of patients with low CD4 count in a study conducted in Central and Western African region[281]. Unexpectedly, a case of HEV1 chronic hepatitis has been recently described in India from a 13-years old child diagnosed with acute lymphoblastic leukemia, indicating a possible involvement in chronic hepatitis of this genotype as well[282].

HEV infections and extrahepatic manifestations

Extrahepatic clinical manifestations during the course HBV or HCV-induced hepatitis are known and involve immune-mediated and inflammation driven multisystemic manifestations. For example, chronic HCV infection can be associated in up to 80% with extrahepatic manifestations such as mixed cryoglobinemia vasculitis, B-cell lymphomas, insulin resistance and glomerulonephritis among others[192,283].

HEV infection may also feature extrahepatic manifestations even with non-clinically overt hepatitis. The range of these manifestations is extended to different organs including mainly central and peripheral nervous system, renal and hematologic system among others[284]. Guillain-Barré syndrome,

meningoencephalitis, glomerulonephritis, cryoglobinemia, hemolytic anemia and pancreatitis have been described with HEV1 and HEV3 infections in both immunocompetent and immunosuppressed patients[285-290]. The virus has rarely been isolated in different organs and hypothetic link of these manifestations to HEV infection is supported by presence of specific signs to affected organs or system and HEV markers with unexplained concomitant elevated liver transaminases (ALT) [289,291-293].

1.3.4. Diagnosis of HEV infection

The diagnosis of HEV acute or chronic hepatitis may require a detection of HEV RNA in serum but this is present for a short period of time. Serological assays for anti-HEV IgM and IgG are commonly used and these antibodies are present during the first months after initial exposure. Anti-HEV IgM is present within the first week of symptoms can be used for diagnosis of acute HEV infection, and anti-HEV IgG for current or past infections. However, seroconversion to both anti-HEV IgM and IgG can be delayed for up to 30 days or more after the first detection of HEV RNA in serum or feces in subclinical cases[294]. Immunosuppressed patients may also have a delayed anti-HEV IgM appearance and the diagnosis of acute HEV infection cannot be based on this marker[295]. Different anti-HEV serological tests are available on the market but they showed variances in sensitivity, specificity and a wide inter-assay discordance[296,297]. Once HEV RNA detected by PCR, HEV genotype can be determined by sequencing the whole genome for phylogenetic analysis[298]. Partial sequencing of ORF1 and/or ORF2/3 can be used for typing already known genotypes[299].

1.3.5. HEV infection prophylaxis and treatment

There is no specific treatment recommended for HEV infection but in severe forms of acute fulminant hepatitis, acute on chronic liver failure, progressive chronic hepatitis and some cases of extrahepatic manifestations, off-label ribavirin has successfully been used and cured the infection in about 80% of the cases[266,286,300,301]. Pegylated interferon alpha associated or not with ribavirin has also been used[278,300]. Immunity acquired from cured HEV infections and from HEV vaccine are protective against a subsequent infection[302]. However, the currently available vaccine is not yet approved worldwide, but it has shown to be effective against HEV1 infections and it is not known if it is effective on other genotypes [303]. Individuals who have cleared infection may loose antibodies within 15 years after a previous exposure and can become again susceptible to HEV new infections[304].

2. AIMS

2.1. GENERAL AIM

The overall objective of this thesis was to evaluate seroprevalence of hepatitis viruses among blood donors and patients and risk factors to get hepatitis infections in Rwanda. The aim was also to perform molecular characterization of these viruses and to determine the circulating genotypes, subtypes, variants and implication of the hepatitis viruses in liver diseases in Rwanda.

2.2. SPECIFIC AIMS

- To evaluate the prevalence of HBV markers and circulating genotypes among Rwandan blood donors (**Paper I**)
- To evaluate the seroprevalence of HCV markers and circulating genotypes among Rwandan blood donors (**Paper II**)
- To evaluate the prevalence of HBV and HCV markers and respective circulating genotypes among liver disease patients and controls and to identify factors associated with the liver disease severity, HBV and HCV infections **(Paper III)**
- To investigate the seroprevalence of HEV and HAV among blood donors, liver disease patients and controls, respective associated factors and circulating genotypes of HEV (**Paper IV)**

3. MATERIALS AND METHODS

3.1. STUDY SITES, PARTICIPANTS AND SAMPLES

We conducted studies for this thesis on volunteer blood donors and on liver disease patients and matched controls. Blood donors were recruited from different regional blood and transfusion centers of Rwanda. Patients with liver disease and controls were enrolled in the study from different hospitals of Rwanda covering the five provinces: Kigali City, Eastern, Northern, Southern and Western Provinces.

In 2015, the Southern and Eastern Provinces accounted for 24.6% and 24.7% of 11.3 million of Rwandan population, respectively. The population in Western province represented 23.5% while that of Northern province and Kigali City accounted for 16.4% and 11% of the national population respectively[30].

Paper I and Paper II

In these two studies, we collected from National Centre for Blood and Transfusion (NCBT) aggregate data on blood donors with regard to numbers of HBsAg and anti-HCV positive donors among first-time and repeat donors, according to gender and residential origin for the year 2014. There were 45,061 blood donations including 13,637 first-time donations and 31,424 repeat donations. We estimated respective prevalence of HBsAg and anti-HCV carriage by calculated ratios among first-time blood donors for HBsAg and anti-HCV reactive over the total number respectively.

We then collected 2ml x 2 of serum from each HBsAg reactive and/or anti-HCV reactive sample available at NCBT. We prospectively collected additional reactive samples obtained from different regional centers and analyzed at NCBT during January and February 2015 to have 240 reactive samples. We collected at

the same time twice as many samples that were negative for both assays. All samples were destined for further analysis at the Laboratory of Clinical Microbiology and Virology (CML) at Sahlgrenska Hospital, Gothenburg, Sweden. Of 720 samples obtained for analysis at CML, 126 were HBsAg reactive, 104 anti-HCV reactive, 10 reactive to both and 480 samples were non-reactive to any of the two assays at NCBT/Rwanda and all were HIV negative (figure 4). These samples were stored at -80°C and were shipped at CML on dry ice and kept at -80°C until they were analyzed.

Paper III

We enrolled 246 patients diagnosed with liver disease by physicians in Rwanda but with no history of any specific antiviral treatment for hepatitis (cases). We simultaneously enrolled 246 patients controls matched to the cases with regard to age (+/- 5 years), gender and province of origin and who were admitted in hospitals or attending outpatient clinics for other clinical conditions than liver disease and who had no known history of liver disease. Both cases and controls were recruited from six different hospitals covering the five different provinces during periods of February-May 2015 and January-June 2016. Of those hospitals, 3 are national referral hospitals: University Teaching Hospital of Butare (CHUB) covering Southern and Western Provinces, University Teaching Hospital of Kigali (CHUK) and Rwanda Military Hospital (RMH) covering Kigali City, Northern and Eastern Provinces. The remaining sites are Kibuye Provincial Hospital located in the Western province, Musanze Provincial Hospital in North and Nyamata District Hospital in the Eastern Province.

Paper IV

In this study, we analyzed for markers for HAV and HEV infection in samples with sufficient materials from participants enrolled in the studies for papers I, II and III (figure4).

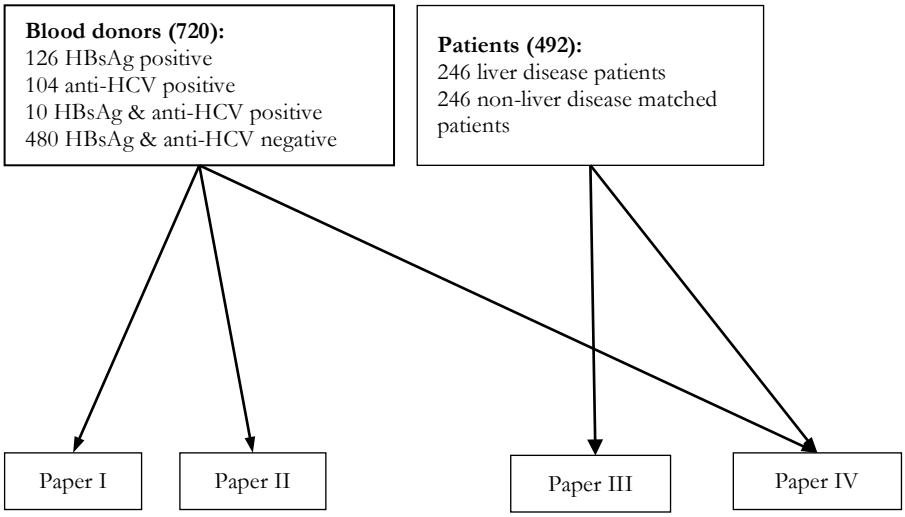


Figure 4. Flow diagram of study participants enrolled in the 4 different studies of this thesis

Data on demographic characteristics and risk factors for HBV and HCV infections

Age, gender, occupation and province of origin were collected for papers II and I in addition to category of donation. The same demographic data were collected for paper III and additional information on risk factors for hepatitis B and C virus infections including sexual orientation, history of unprotected sex with multiple partners; history of sharing sharp objects, history of blood transfusion and/or of invasive procedures in health facilities; history of invasive procedures at home by non-professionals and history of hepatitis or unknown liver disease in a same household as study participant.

3.2. SEROLOGICAL ANALYSIS OF SAMPLES FOR ALL PAPERS

Samples obtained for papers I and II were assayed for anti-HCV, HBV markers and for anti-HDV in HBsAg reactive samples. Samples for paper III were investigated also for serological markers of HCV, HBV and HDV infections

accordingly. HEV serum markers and anti-HAV were performed on blood donors, liver disease patients and their matched controls with sufficient materials for paper IV.

HBV and HDV serum markers

At NCBT, automated chemiluminescent microparticle immunoassays (CMIA) with HBsAg qualitative II by Abbott Laboratories (Abbott Park, IL, USA) on Architect *i*2000R machine was used to investigate HBsAg. At CML, HBsAg assay was repeated together with total anti-HBc, anti-HBc IgM and anti-HBs using CMIA on Architect *i*4000R. HBsAg reactive sera were analyzed for detection of HBeAg and anti-HBe using CMIA assays as well. Also, HBsAg reactive samples and samples with isolated anti-HBc were analyzed for detection of anti-HDV using ETI-AB-DELTA K-2 ELISA kit (Diasorin, Saluggia, Italy).

Anti-HCV serology

Investigations of anti-HCV were performed by CMIA in Rwanda and they were repeated with CMIA on Architect *i*4000R at CML. Samples that were anti-HCV reactive but HCV RNA negative were re-analyzed for anti-HCV by Murex anti-HCV Elisa version 4.0 (Diasorin, Saluggia, Italy). They were additionally subjected to a confirmation test using line immunoassay (INNO-LIA™ HCV Score, Innogenetics, Gent, Belgium). Samples positive or indeterminate with Innolia and samples with detectable HCV RNA were considered as true reactive for anti-HCV and the remaining were considered as false anti-HCV positive[305].

Detection of anti-HAV and anti-HEV antibodies

We used CMIA with anti-HAV IgG kit from Abbott Laboratories (Abbott Park, IL, USA) on Architect *i*4000R to investigate anti-HAV antibodies carriage. We also analyzed samples with sufficient materials for anti-HEV IgG using Enzyme

Linked Immunoassay (ELISA) with HEV IgG third generation kit by DiaPro Diagnostic Bioprobes Srl (DiaPro, Milano, Italy). Given a commonly reported discordance between serological assays for HEV available on market and possible overestimation of anti-HEV IgG by the kit used in this study[295,296,306], we referred to in-house cut point for interpretation of anti-HEV IgG results as follows:

- >1.7 sample signal OD/cut-off OD (S/CO) as anti-HEV IgG positive
- 1-1.7S/CO as borderline anti-HEV IgG or negative
- <1 S/CO as absolutely negative for anti-HEV IgG

We performed analysis for anti-HEV IgM on positive samples for anti-HEV IgG with sufficient materials and on those with borderline results. We assayed anti-HEV IgM on samples from patients diagnosed with acute liver disease in Rwanda irrespective of their anti-HEV IgG status as well by ELISA HEV IgM DiaPro Diagnostic Bioprobes Srl (DiaPro, Milano, Italy). Previously described cut point was used and samples with titers >1 S/CO anti-HEV IgM were considered positive[296,307].

3.3. NUCLEIC ACIDS EXTRACTION AND QUANTITATIVE PCR

Nucleic acids extraction

We performed on 200µL of serum from all samples reactive for HBsAg, isolated anti-HBc, anti-HCV, anti-HEV IgG or IgM and borderline anti-HEV IgG, nucleic acids' extraction by using NucliSENS EasyMAG automated kit and extractor (BioMerieux, Marcy l'Etoile, France). We obtained 110µL of product for each sample eluted in nuclease-free water that was stored at -80°C until further analyses.

Real-time PCR HBV DNA detection

The extracted nucleic acids were analyzed for HBV DNA detection by using TaqMan qPCR assay targeting the S-region with primers HBV305S, HBV460AS and probe HBV-ProbeP previously described[308]. In this reaction, we used 5 µl of extracted DNA, 12.5 µl 2x universal master mix/MgCl2 and Platinum One-Step Quantitative RT-PCR System with ROX (Invitrogen), 0.5 µM of each primer and 0.4 µM probe to obtain a final volume of 25 µl. The reaction was performed on Applied Biosystems 7300 platform (USA) in following conditions: initial denaturation at 50°C for 2 minutes and 95°C for 10 minutes followed by 45 times cyclic reaction at 95°C for 15 sec, 55°C for 15 sec and 72°C for 30 seconds. Cycle threshold (Ct) values obtained for samples were used to estimated viral loads by regression analysis using a standard curve generated by values of serial dilutions 1/10 to 1/100,000 of a sample with known HBV DNA. Nuclease-free water was used as negative control.

Real-time PCR for HCV RNA detection and cDNA synthesis

We also used TaqMan qPCR assay for HCV RNA detection with primers targeting nucleotide positions 180-354 in the 5´UTR of HCV genome. The reaction mix was composed of 20 µl of extracted RNA, 25µl of 2 × universal master mix/MgCl2, 40 U of RNAse OUT (Invitrogen), 200 U of Superscript-III Reverse Transcriptase *Taq* Mix (Invitrogen), 0.1 mM HCVKOD sense primer (5'-CTAGCCGAGTAGYGTTGGGT-3'), 0.4 mM HCVKOD antisense primer (5'-CATGTT GCACGGTCTACG AG-3') and 0.2 mM probe HCVPKOD (FAM-5'CTCGCAAGCACCCTATCAGGCAGTAC-3'-BHQ1) in a final volume of 50 µl. The amplification reaction conditions consisted of initial reverse transcription at 50°C for 45 minutes followed by denaturation at 95°C for 10 minutes. The cyclic reaction was set to 45 cycles with denaturation at 95°C for 30 sec and elongation at 60°C for 60 seconds. Serial dilutions (1/10 to

1/1,000) of a sample with known HCV RNA titer were used as positive controls and nuclease-free water as negative control.

For samples with detectable HCV RNA, cDNA was synthetized for use in PCR amplifications for sequencing. In this reaction, we used 5μl of extracted RNA, 4μg random primers, 0.5 mM of dNTP (Roche), 1× First Strand Buffer (Invitrogen), 5 mM of dithiothreitol (DTT) (Invitrogen), 40 U of RNAse OUT (Invitrogen), 200 U of Superscript III Reverse Transcriptase (Invitrogen) for a total volume of 25 μl. The reaction mix was incubated at 25°C for 10 min then at 50°C for 90 min. The resulting cDNA was stored at-80°C until it was used for the PCRs.

Real-time RT-PCR HEV RNA and cDNA synthesis

In the RT-PCR, the highly conserved overlapping regions in the ORF2/3 of the HEV genome was targeted using forward primer JVHEVF, reverse primer JVHEVR and probe JVHEVP as previously described[236,309]. Synthesis of cDNA was performed by reverse transcription on samples with detected HEV RNA as previously described[236].

3.4. PCR AMPLIFICATIONS AND SEQUENCING

PCR amplification for HBV S-gene

In **paper I**, HBV S-gene was amplified by PCR in a reaction volume mix of 50μl containing 5μl of extracted HBV DNA, 31.9 μl of RNAse-free water (Sigma), 1x Taq buffer (Applied Biosystems), 3 mM $MgCl_2$ (Applied Biosystems), 0.2 mM dNTP, 0.3 mM of each primer, and 1 U of Taq polymerase. We used pooled sense primers gtA1-2792S1-S2 and anti-sense primers gtA1-356AS1-AS2 in a first round PCR. Amplicons from the first PCR were semi-nested in a reaction containing 3μl of the amplicon, primers gtA1-2809S1-S3, the anti-sense primer

and 2.75 mM MgCl2. Additional PCR using pairs of primers HBV-2730S1-8/HBV-874R1-R4 in first PCR, subsequent semi-nested PCR with primers HBV2730S/HBV-98R1-R4 and a PCR using primers HBV3125S1-S6/548R1-R2 and HBV464S1-S2/874R1-R4 were performed to achieve complete S-gene PCRs when necessary. Steps for these PCR reactions are detailed in table 4 and a final step of elongation at 72°C for 10 min was added to each reaction. Primers' sequences are also detailed in table 5.

In **paper III**, the first PCR was performed using primers 2800S/1011AS in a reaction mix containing 5µl of extracted HBV DNA, 1x Taq buffer (Applied Biosystems), 2 mM $MgCl_2$ (Applied Biosystems), 0.25 mM dNTP, 1 mM of each primer, 5 U of Taq polymerase and water to a final volume of 50 µl. Amplicon obtained was semi-nested in 2 PCRs with primers 2800S/P8 and P7/1011AS in conditions presented in table 4. For some samples, PCR amplifications with primers gtA1-2809S1-S3/874R1-R4 and HBV-2730S1-8/548R1-R2 were performed to have a complete S-gene.

PCR amplifications for HCV Core and NS5B genes

First and semi-nested PCRs were performed using HCV cDNA template to amplify 364 and 411 nucleotides of partial NS5B and core regions respectively. The reaction mix contained 5µl of cDNA, 1×Taq buffer (Applied Biosystems), 0.2 mM dNTP, 0.3 mM of each primer cs1/cas1 for core region and ns5bs/ns5bas1 (table 5), 1 U of Taq DNA polymerase, 2.25 mM $MgCl_2$ in a final volume of 50 µl. Products from these first PCRs were semi-nested in PCR using primers cs2/cas1 and ns5b/ns5bas2 respectively (table 5). Steps and conditions for PCRs are also detailed in table 4.

Table 4. Conditions for HBV and HCV PCR amplifications for S-gene, core and NS5B regions respectively

Primers		Initialization		Denaturation		Annealing		Elongation	
		Number of cycles: 40							
		T°	Duration	T°	Duration	T°	Duration	T°	Duration
HBV PCR S-gene									
gtA1-2792S1-S2 / gtA1-356AS1-AS2		95	3min	94	30 sec	60	60 sec	72	60 sec
gtA1-2809S1-S2/ gtA1356AS1-S2		95	3min	94	30 sec	59	60 sec	72	60 sec
HBV-2730S/HBV-874R		95	3min	94	30 sec	59	60 sec	72	60 sec
HBV2730S/HBV-98R		95	3min	94	30 sec	59	60 sec	72	60 sec
HBV3152S/548R		95	3min	94	30 sec	59	60 sec	72	60 sec
HBV464S /875R		95	3min	94	30 sec	59	60 sec	72	60 sec
2800S/1011AS		94	30sec	94	40 sec	59	60 sec	72	60 sec
2800S/P8		94	30sec	94	40 sec	57	60 sec	72	120 sec
P7/1011AS		94	30sec	94	40 sec	55	90 sec	72	60 sec
HCV PCR									
NS5B	ns5bs/ns5bas1	94	3 min	94	30 sec	59	45 sec	72	75 sec
	ns5bs/ns5bas2	94	3 min	94	30 sec	60	45 sec	72	75 sec
Core	cs1/cas1	94	3 min	94	30 sec	61	45 sec	72	75 sec
	cs2/cas1	94	3 min	94	30 sec	61	45 sec	72	75 sec

PCR amplifications for partial RdRp domain on HEV ORF1 and on ORF2/3

First PCR on partial ORF1 was performed on HEV cDNA by using pooled forward primers ISP4232 and reverse primers EAP4576 and the amplicon was semi-nested in a PCR using pooled primers IASP4561 and EAP4576 as previously described[236]. We also performed PCR amplifications of partial ORF2/3 overlapping regions using sense primer HE361 and anti-sense primer HEV364 as previously described[310]. PCR amplicons were visualized on ethidium bromide-containing gel agarose electrophoresis 3% before any attempt of sequencing.

PCR amplicons were purified and extracted by using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. A sample of 5.4 μL of each purified product, 1.6 μM of each primer used in nested PCR and 2μl of Terminator Ready Reaction Mix and 1μl of sequencing buffer were used for cycle PCR sequencing reactions. The products were sequenced using fluorescent-labeled dideoxy-chain terminators and reagents in the BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems). Analyses were performed on the 3130xl Genetic Analyzer (Applied Biosystems).

3.5. GENOTYPE AND SUBTYPE DETERMINATION BY PHYLOGENETIC ANALYSIS

Respective sequencing products were analyzed in the SeqMan section of DNAStar programme package version 10.1.2 (DNA Star Inc, Madison, WI 53705, USA). Sequences of the S-gene were aligned with corresponding region of sequences representing HBV genotypes obtained from GenBank, including all HBV genotype A strains from Africa. Likewise, sequencing products for HCV were aligned with available sequences for HCV NS5B and core regions of HCV genotype 4 strains originating from Africa, Middle East, Western Europe and Northern America obtained from the GenBank as well. Phylogenetic analysis was carried out with the PHYLIP package version 3.65. Evolutionary distances were calculated using the F84 algorithm in the DNADIST program. Unweight pair-group method using arithmetic averages (UPGMA) and the neighbor-joining method in the NEIGHBOR program, PHYLIP package was used to construct the phylogenetic trees, which were visualized using the program Tree View, version1.6.6.

Table 5. List of primers used for HBV and HCV amplification and sequencing

Virus	Region	Name	Nucleotides' sequence (5'-3')
		2800S	CAGGTAGCGCCTCATTTTGTGGGTCACCATATTCT
		1011AS	CAAAAGACCCACAATTCTTTGACATACTTTCCAAT
		P8	GTGGTGGACTTCTCTCAATTTTC
		P7	CGGTAWAAAGGGACTCAMGAT
		gtA1-2792S1	GGAAACTACACGTAGCGCCTCATTTTG
		gtA1-2792S2	GGAAACTACACGTAGCGCTTCATTTTG
		gtA1-356AS1	ATTTGTCCTGGTTATCGCTGGATGTGT
		gtA1-356AS2	ATTTGTCCTGGTTATCGCTGGATGTGT
		gtA1-2809S1	CCTCATTTTGCGGGTCACCATATTCTT
		gtA1-2809S2	CCTCATTTTGCGGGTCACCATACTCTT
		gtA1-2809S3	CTTCATTTTGCGGGTCACCATATTCTT
		HBV-2730S1	CATTACTYCCAGACCCGACATTATTTACAT
		HBV-2730S2	CATTACTTCCAGACCCGACACTATTTACAT
		HBV-2730S3	CATTACTTCCAGACCCGACATTATTTGCAT
		HBV-2730S4	CATTACTTCCAGACCCGACATTAATTACAT
		HBV-2730S5	CATTACTCCCAGACCCGACCTTATTAACAT
		HBV-2730S6	CATTACTTCCAAACCCGACATTATTTACAT
		HBV-2730S7	CATGAGGCCCAGACCCGACCTTATATACAT
		HBV-2730S8	CATTACTTCCAGACTCGACATTATTTACAT
		HBV-874R1	ACCCCAACTTCCAATTACATAKCCCATGAA
		HBV-874R2	RCCCCAACTTCCAATTATRTATCCCATGAA
		HBV-874R3	ACCCCTACTTCAAATTACRTATCCCaTGAA
HBV	**S-gene**	HBV-874R4	GCCCCAaCTTCCAATYACATATCCcATGAA
		HBV-548R1	TTTTGTACAGCAACATGRGGGAAACATAGA
		HBV-548R2	TTTTGTACAGCAACATGAGGGAATCATAGA
		HBV-3125S1	AGTCAGGAAGGCMGCCTACTCCCAT
		HBV-3125S2	AGACAGGGAGGCAGCCTACTCCCAT
		HBV-3125S3	CCTATCGAAGGCAGCCTACTCCCAT
		HBV-3125S4	AGGCAGGAAGGCAGCCTACTCCCAT
		HBV-3125S5	AGTCACGTAGGCAGCCTACTCSCAT
		HBV-3125S6	AGTCAAGGAGGCAGCCCACTCCCAT
		HBV-98R1	TTGACGAGATGTGAGAGGCARTATT
		HBV-98R2	TTGACGATATGTGAGAGACAATATT
		HBV-98R3	TTGACGATATGTGAGAGGCAATATT
		HBV-98R4	TGGAGGAGATGGGAGAGGCAATATT
		HBV-464S1	TTGCCCGTTTGTCCTCTAATTCCAGGATCC
		HBV-464S2	TTGCCCGTTTGTCCTCTSATTCCAGGATCC
		CS1	ACTGCCTGATAGGGTGCTTGC
	Core	CS2	AGGTCTCGTAGACCGTGCA
HCV		CAS1	ATGTACCCCATGAGGTCGGC
		NS5BS	TATGAYACCCGYTGCTTTGAC
	NS4B	NS5BAS	GAGGAGCAAGATGTTATCAGCTC
		NS5BAS2	GAATACCTGGTCATAGCCTCCG

3.6. STATISTICAL ANALYSIS

Prevalence of respective HBV, HCV, HEV and HAV markers in different groups were presented as percentages. We performed comparisons of prevalence for HBsAg, anti-HCV and HCV subtypes between different groups using chi-square or Fisher exact tests accordingly. Odds ratio (OR) and 95% confidence intervals (CI) were provided where applicable. In papers III and IV, factors achieving a significant difference ($p<0.25$) in the univariate analysis were used in multivariable logistic regression analyses to identify independent factors to respective current infection or past exposure to HBV, HCV, HEV and anti-HAV accordingly. In paper III, we controlled for age in model for comparison of prevalence of HCV subtypes according to severity of liver disease. All analyses were performed using SPSS version 24.0 (Chicago, IL) and a $p< 0.05$ was considered statistically significant.

3.7. ETHICAL CONSIDERATIONS

The Rwanda National Ethics Committee approved the study (No 243/RNEC/2014) and the Institutional Review Board of the College of Medicine and Health Sciences at University of Rwanda (IRB/CMHS-UR) extended that approval to 2016. Participants were at least 18 years old and provided informed consents when applicable.

RESULTS AND DISCUSSION

4.1. PAPER I: Prevalence and characterization of HBV markers among blood donors in Rwanda

4.1.1. HBsAg prevalence

In Paper I, we investigated prevalence of past or active HBV infection in Rwandan blood donors. Previous estimates were based on small numbers of study participants or studies conducted on specific groups of patients, mainly HIV patients and pregnant women attending urban clinics[33,34]. In 2010, Rwanda was classified among countries with intermediate endemicity of HBV with an estimated HBsAg prevalence of 6.7% diverging with results from those small studies on HIV-infected pregnant women conducted in 2007 and on HIV patients carried in 2013 in Kigali city, from which prevalence of 2.4% and 5.2% respectively were found[4,5,33,34]. Results from our study on HBsAg prevalence among first-time blood donors have confirmed the intermediate endemicity of HBV infection with an estimated nationwide HBsAg prevalence of 4.1% in the year 2014[311]. Although, the prevalence of markers for past or present HBV infections in blood donors may be of moderate significance for population estimates, the financial affordability, especially in resources restricted areas, makes them attractive as the cheapest way to access to the prevalence for healthy populations. In the context of Rwanda where the majority of the population is not aware of its HBV serological status, prevalence among first-time donors is a reasonable approximation of the prevalence in the adult population but may need adjustments for age and gender[312,313]. Since acute and chronic HBV infections are mainly asymptomatic, the majority of the first-time blood donors were unaware of their status and the risk to exclude an important proportion of infected individuals during pre-donation screening that could lead to an underestimation of the prevalence was then minimal [314]. We could not get from NCBT data on age-specific HBsAg prevalence and the age distribution of

blood donors to adjust the overall prevalence found. Despite that, results from this study give added value as representatives from different regions of the country were included contrarily to previous studies.

However, our results may somehow overestimate the general prevalence. We found the general prevalence among first time at 4.1%, but it was 5.1% among male first-time donors and significantly higher than 2.4% found among female first-time donors ($p<0.001$, Fisher's exact test) while females represented only 36.7% of first-time donors. This gender difference corroborates findings from other African countries from which male gender was found as an independent risk factor for HBV infection[315-318]. Hormonal effects on progression to chronic hepatitis B can be one explanation to this. It has been shown that estrogens have down-regulatory effects on HBV pgRNA transcription and HBV DNA replication while androgens have opposite effects [319].

4.1.2. Prevalence of markers for previous exposure to HBV

From analyses performed at CML on 581 HBsAg negative blood donors including 105 females and 476 males, we found that 122 (21%) had both anti-HBc and anti-HBs indicating a resolved HBV infection with post-infection acquired immunity. Of those with these markers, 19 were females (18.1%) and 103 (22.3%) were males and there was no significant difference with regard to gender ($p=0.362$; Fisher's exact test). Fourteen of the 581(2.4%), all males, had isolated anti-HBc indicating cured or occult HBV infection as we did not find detectable HBV DNA in any of these samples. The sum of those with anti-HBc and those with HBsAg, we showed that 27.5% of the Rwandan blood donors had at least one marker of active or cured HBV infection. This prevalence was relatively lower compared to that from neighboring countries like Uganda and Kenya where anti-HBc seroprevalence exceeded 50% while the highest reported in Rwanda was 42.9% among HIV patients[34,320].

This indicates that more than 68% of adults in Rwanda may benefit from HBV immunization since we found also that few adults have been vaccinated with only 4.3% of the blood donors in this study having isolated anti-HBs, a marker of immunity acquired from vaccination against HBV. This low vaccination coverage in adults was confirmed in a study on healthcare workers in Rwanda carried out in 2013, which showed that only 4.5% of interviewed participants reported having had the vaccine[321]. The universal vaccination against HBV is part of the Extended Program of Immunization for all children in Rwanda since 2003. The vaccination coverage was recently estimated at 98% among children. However, there is a need to expand this to adults, or at least to groups at risk, in effort to stop new HBV infections[31,322].

4.1.3. HBeAg and anti-HBe prevalence among HBsAg reactive donors

Among different routes of transmission for HBV, it has been shown that perinatal transmission is not as important in SSA countries as in South East Asian countries[323]. High viral load and HBeAg positive status among HBV-infected childbearing mothers are well recognized important factors for the mother-to-child transmission[324]. In this study, the overall prevalence of HBeAg among HBsAg positive blood donors was 24.2%, of which 97% were HBV DNA positive. This HBeAg positive status was more important in blood donors younger than 25 years with a prevalence of 35.7% while less than 4% of the participants older than 35 years had HBeAg. No significant difference of HBeAg status between the genders was found.

The prevalence of HBeAg found in our study was in range with reports from other African countries and was similar to that found among HIV pregnant women in Kigali city [33,322,325]. The prevalence was, however, relatively lower compared to what is reported from Asian countries where it was over 40% for patients younger than 25 years[326]. It has been shown that an earlier clearance of

HBeAg is more pronounced in HBV strains of genotype A, mostly prevalent in Africa [327]. In contrast, HBV genotype C strains that are predominant in South East Asian countries have rather the slowest clearance of HBeAg. HBeAg positive status among pregnant women, by the possibility of the antigen to cross the placenta, and the associated high viral load, predispose children born from HBeAg positive infected mothers to get infected during birth [99,140]. The vertical transmission is thus less important in Rwanda and probably in other African countries, for that reason.

Although HBeAg positive status refers to a high viral load and clearance of HBeAg to a low viral load, relatively high HBV DNA levels may be found in HBV-infected individuals harboring anti-HBe[88]. In this study, we found 49% viraemic samples while anti-HBe positive and 17% of them had an HBV DNA level > 4 $\log_{10}$ IU/ml. The high viral load in absence of HBeAg raises a possibility for presence of HBV strains in Rwanda expressing mutations in PC region or the BCP region[328]. In line with patients harboring HBeAg and its high risk of HBV transmission to the offspring during birth, also neonates born from infected mothers without HBeAg but with a high viral load bear a risk estimated to 23% of getting infected during birth compared with up to 90% for those born from mothers with HBeAg positive status. Moreover, the absence of HBeAg, and its immunomodulatory effects in particular, predispose to massive liver damage of infected hepatocytes by the immune system. This can lead to severe forms liver disease in children such as acute fulminant hepatitis and early progression to LC or to HCC that can occur following the chronic cellular turnover [64,324,329,330]. Infection by strains with mutations abolishing the expression of HBeAg can also predispose to LC and HCC in chronically HBV infected adults and the prevalence of these mutations may vary with prevailing genotypes[64,330].

4.1.4. Circulating HBV genotypes and mutations in the S-gene

Predominance of subgenotype A1

Of 85 samples with HBV DNA, 58 could be sequenced in the S-gene and all clustered within genotype A, subgenotype A1. Moreover, 53 of them formed an isolated sub-clade with other strains previously published from Rwanda and some strains from Belgium, a country that shares a colonial history with Rwanda and that hosts an important proportion of immigrants from Rwanda. Few strains from neighboring countries like Democratic Republic of Congo and Tanzania were also within that clade[311]. The predominance of subgenotype A1 was confirmed in the Central and East African Region but subgenotype A3 and genotype D have also been identified though in relatively small proportions, but we did not find any of them in this study[35,331-334].

It is indicated that strains of HBV subgenotype A1 were originated from Africa and have spread in some Asian countries like India and Latin American countries including Haiti through migrations and trades with the Central African region[335,336]. However, the limited variability of strains isolated in Rwanda indicates that it was rather recently introduced in the country and spread in the different regions of Rwanda almost without any newly introduced strain.

Pre-S deletions

By analyzing sequencing of the S-gene from different strains, we found deletions of different sizes in number of amino acids (aa) within the pre-S regions in 31% of 58 strains sequenced. There was predominantly a deletion of 18 aa in the N-terminal region of the pre-S2 between residues 5 and 22 that was identified in 12 strains. Other relatively smaller deletions of 9, 11 and 14 aa were observed in 3 different strains. We did not find any factor associated with these pre-S2 deletions. The HBeAg status, gender distribution, category of donors and viral

load were comparable between samples containing strains with deletions and those without. Two large deletions involving C-terminal part of the pre-S1region were also found in two strains between amino acids 30 and 84 and between amino acids 67 and 86.

These two large in-frame pre-S deletions may occur spontaneously or be triggered by immunotherapy or antiviral treatment. They occurred in a region overlapping the spacer domain of the Pol gene, which tolerates mutations, to be selected *in vivo*. Likewise, strains with partial pre-S2 deletions could also be selected because these deletions do not impact on the formation, secretion or infectivity of the virion [337]. However, presence of deletions may impact on the severity of the liver disease. Large deletions over pre-S1 and pre-S2 have been associated with a defective secretion of the L mutant envelope proteins and to their accumulation in the ER of infected hepatocytes. This causes oxidative stress, DNA damage and degeneration of hepatocytes into "ground glass" hepatocytes type II that are potentially carcinogenetic[74,338,339]. With this oncogenic potentiality, identification of strains with pre-S2 deletions have been suggested to serve as a tool for prediction of progression to HCC[74]. Pre-S deletions have actually been identified as cause early development of HCC since they were found in samples from children with HBV-induced hepatocellular carcinoma in Asia[340,341]. This deletion being highly prevalent in HBV strains of genotypes B, C and subgenotype A1, the relatively high frequency of deletions found in our study can be one of the hypothesis to an increased rate of HBV-induced HCC at young age in SSA region but further works are needed to confirm or refute this hypothesis[37,107,108].

4.2. PAPER II: Prevalence of anti-HCV and circulating HCV genotypes among blood donors in Rwanda

4.2.1. Anti-HCV prevalence among blood donors

The main goal for this study was to estimate the prevalence of anti-HCV among blood donors and to perform a phylogenetic characterization of isolated HCV strains. Previous estimates and results from studies performed prior to 2014 on HIV patients in Rwanda have shown an anti-HCV prevalence of 4.9-5.7%[10,33,34]. In the present study, the anti-HCV prevalence found was 1.6% among first-time blood donors and it was 0.3% among repeat donors[342]. Although it might be lower compared to the previous reports, we found it somehow overestimated when we re-analyzed the anti-HCV reactive samples by CMIA, Elisa 3rd generation and confirmatory assays as Innolia HCV tests and real-time PCR at CML Gothenburg. Of 120 samples initially anti-HCV reactive in Rwanda, 82 were repeatedly reactive on CMIA assay at CML. However, only 14 of these (17.1%) had detectable HCV RNA. Of the remaining, 11 (9.2%) were positive while 15 (12.5%) were indeterminate with Innolia assay. In summary, there were only 40/120 (33.3%) anti-HCV initially reactive samples that were confirmed true anti-HCV reactive (table 6). The remaining samples were categorized as yielding false positive reactivity for anti-HCV. This high rate of false positive for anti-HCV on Elisa-based assays has been reported elsewhere in the world but the highest rates were found in low and middle-income countries of sub-Saharan Africa[202,343-348]. Therefore, anti-HCV seroprevalence reported from those resource-limited settings may have been overestimated including those from Rwanda as no confirmatory tests were performed in previous studies[349].

Besides the overestimation of anti-HCV by ELISA and/or CMIA assays among the blood donors, the seroprevalence of anti-HCV in this group might also be a poor estimate of anti-HCV for the general population in Rwanda. We have found this study for instance that the seroprevalence was somehow higher in

females and in donors older than 40 years. These particular groups are less likely to donate blood and we can assume that observed prevalence might be inferior to real rates in general adult population in Rwanda[350]. In a study conducted in 2016 on HIV-infected individuals in Rwanda, the overall prevalence of anti-HCV by ELISA was 4.6%. However, a gradually increasing rate with age was observed. The prevalence was 1.7% in participants younger than15 years and it reached 17.8% in patients older than 65 years[351]. This age-related anti-HCV seroprevalence was also confirmed in another small study conducted on patients attending the department of Medicine for day care in one hospital in Rwanda[352]. Likewise, in **paper III** of this thesis, we found old age and female gender as independent risk factors to HCV infection among others. This higher anti-HCV prevalence in older generations in Rwanda has probably to do with history of previous exposure to unsafe invasive procedures at home, within health facilities or shared blood at occasion of ancient ritual practices, quite common up to the last decades of the 19th century. It is with the discovery of HIV/AIDS and the awareness of its transmission routes that are shared with the blood-borne hepatitis viruses that has boosted preventive measures and probably allowed a decrease of HCV exposure and of anti-HCV prevalence in younger groups in Rwanda [353].

In an effort to decrease such high rates of false positive anti-HCV among blood donors that certainly lead to unnecessary discard of blood products, psychological stress and unnecessary expenses to donors falsely diagnosed with possible HCV infection, specific strategies are needed at NCBT-Rwanda. The most affordable solution to the situation could be to identify a cut-off point that separates false positive from true positive anti-HCV as previously recommended[354]. However, the preconized cut-off of 5 S/CO for CMIA assays on Architect could not stand according to the results from our study. Three samples with anti-HCV titers lower than this recommended cut-off were confirmed true positive by Innolia although none was HCV RNA positive[354].

Table 6. Anti-HCV reactivity by CMIA assay, Innolia and HCV RNA

	Anti-HCV+ in Rwanda N	Anti-HCV+ in Sweden N	True anti-HCV+ HCV Innolia indeterminate N	HCV Innolia positive N	HCV RNA positive N	Total of true Anti-HCV+ N (%)
Province						
East	41	29	6	6	3	15 (37)
Kigali	3	2	0	0	0	0 (0)
North	11	10	1	0	3	4 (36)
South	53	32	6	4	5	15 (28)
West	12	9	2	1	3	6 (50)
Age group (years)						
19-29	72	44	12	8	6	26 (36)
30-39	37	29	3	2	4	9 (24)
40-60	11	9	0	1	4	5 (45)
Sex						
Male	95	62	9	8	10	27 (28)
Female	25	20	6	3	4	13 (52)
Category of donors						
First time	82	59	11	10	13	34 (41)
Repeat	38	23	4	1	1	6 (16)
TOTAL	**120**	**82**	**15**	**11**	**14**	**40 (33)**

However, the HCV RNA negative status could be interpreted differently. The samples are either from donors with cured HCV infection or with HCV RNA titers below the limit of detection. Admission of these products with HCV RNA under detection threshold could transmit HCV infection. A standard practice could be the investigation of HCV RNA on all anti-HCV reactive samples but this test might not only be time-consuming but also expensive for a resource-limited country like Rwanda. Analysis by using HCV core Ag assay can be an attractive alternative to HCV RNA although this also remains less sensitive[205,355]. However, in case the HCV core Ag assay is opted for, negative samples for this assay but anti-HCV positive can be additionally analyzed by line immunoblot assay to address the challenges of the volunteer donors and of the number of blood products unduly discarded[345,356,357]. Line immunoblot assays are based on recombinant proteins derived from HCV, which are generally broader than those used in enzyme immunoassays and false positive results quite very rare[305,347,357].

4.2.2. Circulating HCV genotypes among blood donors

All 14 HCV RNA positive samples could be sequenced in partial core region and 13 were sequenced in partial NS5B region. All but one belonged to HCV genotype 4 including 6 strains clustering within subtype 4k, 3 in subtype 4r, 2 in subtype 4q and 2 could not be classified in any of so far defined subtype from NS5B sequences[149]. One strain was clustering within subtype 4r when the core gene was analyzed and within subtype 4q with the NS5B region analysis and could have been typed as an intra-genotypic recombinant 4q/r. However, we could not sequence the whole genome to exclude a possible dual infection by strains belonging to the two different subtypes or to identify the crossover point for the potential recombination.

The predominance of HCV genotype 4 is relatively common in the Central African region where Rwanda is located as similar predominance of genotype 4 have been reported from Uganda, Burundi and Democratic Republic of Congo[358-360]. However, subtypes 4k that are highly prevalent in Rwanda were only found predominant in the Democratic Republic of Congo where they formed with subtypes 4c the major strains isolated in that country. In Burundi and Uganda, subtypes 4h and 4a respectively accounted for majority of strains to show how divergent are HCV strains in that region[358-360]. Similarly, in a small study conducted in Nairobi, Kenya, authors did not find any strain of genotype 4 but rather a predominance of genotype 2, subtype 2b[346]. Also, genotype 1, 2 and 3 have been described in less than 2% of patients treated for hepatitis C infection in one private hospital in Rwanda but there is no further description neither on methods used for sequencing nor on the origin of those patients mentioned in that short report. These genotypes have actually been found in neighboring countries as Uganda and Kenya[36,358,361]. In the present study, we did not find any strain belonging to genotype 1 or 2 and it was also the case in study for **paper III** with much larger sample size performed on liver disease patients. One case, a young blood donor living in Western province, in this study for **paper II** was infected with HCV genotype 3 strain, subtype 3h. This strain was phylogenetically close to strains prevailing in Somalia and Middle East [362].

4.3. PAPER III: Implication of HBV and HCV in liver disease in Rwanda and their respective risk factors

This study aimed to evaluate the contribution of HBV and HCV to liver diseases and to their severity in Rwanda. Relatively recent studies conducted in resource-limited areas and in developed countries concurred on that prevalence for both HBsAg and anti-HCV in patients with liver disease exceeds 50%. There are however, geographical differences with regard to the importance of the contribution of those viruses and to the predominance of one virus compared to the other[1,14,114,363,364]. HBV has been commonly reported as more predominant than HCV in SSA countries contrasting with current situation in developed nations[195]. However, relatively old studies from the SSA region have in some countries revealed a predominance of HCV in chronic liver disease and HCC but these data need to be updated[203,365].

4.3.1. Prevalence of HBsAg and anti-HCV among cases and controls

In this study for **paper III**, we enrolled 246 patients diagnosed with liver disease (cases) in Rwanda and 246 controls matched with regard to age, gender and origin. There were 125 females (50.8%) and 121 males (49.2%) among cases and controls. We found both HBsAg and anti-HCV prevalent in 74% (182/246) of cases with liver disease and in 17% (42/246) of controls ($p=0.002$). A dual HBV-HCV infection was diagnosed in four (1.6%) patients with liver disease but in none among the controls. In both cases and controls, anti-HCV was more prevalent than HBsAg. There were 109/246 (44.3%) anti-HCV reactive samples from patients with liver disease and 33/246 (13.4%) from controls. HBsAg was prevalent in 77/246 (31.3%) of cases and in 9/246 (3.7%) of controls ($p<0.001$, tables 2, **paper III**).

4.3.2. Risk factors to HBV infection or exposure

Cases and controls with active HBV infection were much younger than anti-HCV positive study participants. The mean age for HBsAg carriers was 39.1+/-14.6 years versus 59.1+/-15.5 years for anti-HCV positive patients. It was 38.6+/-12.2 years for HBsAg positive versus 63.2 +/- 14.5 years for anti-HCV positive among controls (table 2, paper III). Likewise, the table 7 of this thesis shows that among cases who were younger than 49 years, 62 (50.4%) were HBsAg positive compared to 15(12.2%) in the elder group ($p<0.001$; Fisher's exact test). Table 7 also shows a gender difference with regard to HBsAg carriage. Males were more represented among HBsAg reactive cases than females. Forty-nine (40.5%) males were HBsAg positive compared to 28 (22.4%) females ($p=0.002$; Fisher's exact test). Among the controls, nine participants had detectable HBsAg, five were males and four were females ($p=0.960$; Chi-square with Yates correction).

In a multivariable logistic regression analysis controlling for age, gender and other risk factors for HBV infection, male gender tended to be more associated with HBsAg carriage (OR =1.54[0.92-2.61]; $p=0.097$; table 8) and so did the young age (OR=0.96[0.94-0.97]; $p<0.001$; table 8). However, this trend was not found when we performed the analysis for anti-HBc, a marker of exposure to HBV. Likewise, there was no association of age with anti-HBc serological status (table 8).

This association of male gender and active HBV infection (HBsAg) contrasting with finding when anti-HBc markers is used in the analysis is an indication that HBV-infected males tend to progress to chronic hepatitis B more often than females. An explanation to this may be linked to the protective effects of estrogens for childbearing and premenopausal aged females from progression of the HBV infection to chronicity while androgens exert opposite effects[366,367]

Table 7. Distribution of cases and controls according to anti-HCV and HBsAg prevalence and with regard to age and gender

	Cases						**Controls**					
	Males			**Females**			**Males**			**Females**		
Age group	**N**	**HBsAg +**	**Anti-HCV+**	**N**	**HBsAg +**	**Anti-HCV+**	**N**	**HBsAg +**	**Anti-HCV+**	**N**	**HBsAg +**	**Anti-HCV+**
19-28	**21**	11(52.4)	4 (19.1)	**15**	7(46.7)	3(20.0)	**23**	1(4.3)	0 (0.0)	**14**	0(0.0)	0(0.0)
29-38	**24**	17(70.8)	4(16.7)	**20**	8(40.0)	5(25.0)	**29**	3(10.3)	2 (6.9)	**20**	2(10.0)	0(0.0)
39-48	**25**	13(52.0)	2(8.0)	**18**	6(33.3)	6(33.3)	**21**	0(0.0)	2(9.5)	**22**	1(4.6)	3(13.6)
49-58	**18**	3(16.7)	8 (44.4)	**21**	4(19.1)	14(66.7)	**18**	1(5.6)	1(5.6)	**20**	0(0.0)	1(5.0)
59-68	**19**	3(15.8)	11(57.9)	**22**	1(4.5)	19(86.4)	**14**	0(0.0)	0(0.0)	**23**	1(4.3)	10(43.5)
69-78	**7**	1(14.3)	3(42.9)	**25**	1(4.0)	22(88.0)	**10**	0(0.0)	2(20.0)	**20**	0(0.0)	6(30.0)
>79	**7**	1(14.3)	5(71.4)	**4**	1(25.0)	3(75.0)	**6**	0(0.0)	2(33.3)	**6**	0(0.0)	4(66.7)
Total	**121**	49(40.5)	37(30.6)	**125**	28(22.4)	72(57.6)	**121**	5(4.1)	9 (7.4)	**125**	4(3.2)	24(19.2)

It has been found that androgens have such effects by upregulating HBV enhancer I expression, which increases the HBV mRNA transcription and HBV DNA replication in mice at puberty. Castration of these mice and administration of estrogens or anti-androgens have shown opposite regulatory effects[319,368]. Another potential mechanism could rely on effects of enhancement by estrogens, and estradiol in particular, on immune cells that may result in more clearance of infectious pathogens including HBV with resulting protection of HBV-infected childbearing females from progression to chronic hepatitis B[369,370].

We also found that history of multiple sex partners was an independent factor to both anti-HBc and HBsAg (table 8). This confirms the sexual transmission as one route for HBV infection in Rwanda on top of other horizontal transmission

routes that are generally common in SSA countries such as shared blood via micro-traumatic lesions during childhood and iatrogenic transmissions[323,371,372].

Table 8. Factors to active and/or past HBV infection, HCV infection: summary of logistic regression analysis with 5 covariates

	HBsAg		Anti-HBc		Anti-HCV	
Factors	**Adj. OR [95%CI]**	**p-value**	**Adj. OR [95%CI]**	**p-value**	**Adj. OR [95%CI]**	**p-value**
Age (in years)	0.96[0.94-0.97]	<0.001	1.00[0.99-1.01]	0.960	1.07[1.05-1.09]	<0.001
Male gender	1.54[0.92-2.61]	0.097	1.19[0.81-1.74]	0.376	0.47[0.29-0.75]	0.002
Multiple sex partners	1.67[0.99-2.82]	0.055	1.92[1.29-2.85]	0.001	1.04[0.63-1.71]	0.882
History of sharing sharp objects	0.94[0.75-2.18]	0.371	0.98[0.67-1.45]	0.931	1.16[0.72-1.86]	0.549
History of liver disease in same household	1.62[0.95-2.77]	0.075	1.06[0.69-1.61]	0.795	2.23[1.34-3.70]	0.002

4.3.3. Risk factors to HCV infection/exposure

Age and prevalence of anti-HCV

We found that anti-HCV reactive liver disease patients and controls were significantly older than nonreactive participants ($p<0.001$; table 8). There were 85/123 (69.1%) patients with liver disease older than 48 years who were anti-HCV reactive compared to only 24/123 (19.5%) in those younger than 49 years ($p<0.0001$; Fisher's exact test, table 7). There was also an increasing rate for anti-HCV carriage with age among the controls. The prevalence was 5.5% in participants younger than 49 years versus 22.2% in the older group ($p=0.0001$; Fisher's exact test, table 7).

Our results concur with findings from recent studies conducted in Rwanda on HIV patients and patients consulting for medical care in one hospital in Kigali city that have shown an age-dependent prevalence for anti-HCV as well[351,352].

Gender and anti-HCV prevalence

We found in this study that female participants were more likely to be HCV infected than males. Among females with liver disease, 72/125 (57.6%) were anti-HCV reactive compared with 37/121 (30.6%) males ($p<0.0001$; Fisher's exact test; table 7). This predominance of female gender among HCV-infected was also observed among controls with 19.2% of female controls being anti-HCV reactive versus 7.4% in males ($p=0.007$; Fisher's exact test; table 7).

HCV infection and history of liver disease in same household

More patients with liver disease reported a history of hepatitis in the same household than controls (30.9% vs. 18.7%; $p=0.002$; table 1 of **paper III**). Also, anti-HCV carriers reported more this history than anti-HCV nonreactive participants. There was also a trend of association of that history and HBsAg (table 8). In a multivariable logistic regression to investigate for the effect of age on HCV infection when adjusting for gender, history of multiple sex partners, history of shared sharp objects and history of liver disease in same household, we found increased age, female gender and history of liver disease in same household as independent factors for HCV infection.

The mechanisms by which individuals have acquired HCV infection and vehicles of the current spread in SSA region and in Rwanda in particular remain not well understood[10]. In a study on blood donors in Ghana, Layden *et al* found tribal scarifications, circumcision at home and home birth as independent factors to HCV infection among other factors[174]. Iatrogenic and intra-familial transmissions of HCV were also stressed in a study conducted in Cairo, Egypt[373]. Similar mechanisms may explain the transmission of HCV in Rwanda.

Scarifications and customs of sharing blood, invasive procedures by non-professional healthcare providers at home for teeth extractions, intramuscular injections with reuse of unsterile needles and deliveries at home were quite common until recently when HIV/AIDS pandemic was discovered[353]. The age-dependent HCV prevalence in our study could find an explanation in the decreasing prevalence of unsafe practices in the country since that period. Females may have been particularly at higher risk than males through the multiple occasions for invasive procedures at home during deliveries. Moreover, they may have been exposed to iatrogenic transmission in health facilities by systematic immunizations during pregnancies or by invasive procedures during deliveries with use of unsterile materials.

Both HBV and HCV are more likely to be acquired when a family member in the same household has been infected. This risk for both HBV and HCV transmissions have also been described in the SSA region and among blood donors in USA[315,323,374].

Our results foster a systematic screening for HCV for not only individuals older than 50 years but also those whose family member is HCV-infected in Rwanda. The latter is also applicable for screening of HBV infection. In contrast, HBV screening should rather focus on young generations when family history is uneventful.

4.3.4. HBV and HCV markers at different stages of liver disease

We found a predominance of HCV-infected individuals among patients with advanced liver disease. Of 90 patients diagnosed with advanced liver disease including 72 cases of liver cirrhosis and 18 cases of HCC, there were 42 (58.3%) HCV-infected versus 13 (18.1%) HBV-infected ($p=0.002$; Fischer's exact test) among patients with LC, and eight HCV-infected (44.4%) versus two (20%) HBsAg carriers among those diagnosed with HCC (table 2, **paper III**). These

findings on predominance of HCV infection among patients with advanced liver disease confirmed results of a study conducted from Rwanda in 1992[203]. Moreover, HCV-infected patients with advanced liver disease were more likely to be older and accounted more females than the HBV-infected ones (table 2, **paper III**).

Notwithstanding the HCV predominance among liver disease patients, of 141/246 (57.3%) diagnosed with chronic liver disease, 58 (41.1%) and 56 (39.7%) were HBsAg and anti-HCV carriers respectively. Also, HBsAg carriers were much younger and accounted for more males than HCV-infected patients (tables 2 &3, **paper III**). This highlights again the predominance of HCV infection in liver disease in Rwanda but more specifically in advanced forms of cirrhosis and HCC. Such pattern is mainly reported in developed countries and might indicate that the implication of HCV in liver disease is probably underestimated in other SSA countries[194,195,312,375]. However, in Central and Western African countries highly endemic for HBV, where HDV is also endemic, the contribution of HBV in both advanced and non-advanced liver disease may supersede that of HCV [14,114]. In some of countries, patients with HCV-induced HCC were older than patients with HBV-induced HCC probably due to the same age-cohort effect as that remarked in Rwanda and in developed nations[14].

The results in this study have particular importance for healthcare professionals as they may serve for guidance in elaboration of strategies for prevention and advocacy for HCV infections' treatment since they can nowadays be completely cured. In addition, identification of HCV circulating genotypes is a useful step for selection of effective therapeutic anti-HCV regimen.

4.3.5. HCV genotypes/subtypes and phylogenetic analysis

Of 91 HCV RNA positive samples from patients with liver disease and 19 samples from controls, 88 (96.7%) and 16 (84.2%) could respectively be sequenced. All but one belonged to the genotype 4 and the majority of them or 44.2% were clustering within subtype 4k, while 23.1% and 17.3% belonged to subtypes 4r and 4q respectively. One sample from a patient with liver disease clustered within genotype 3, subtype 3a. The overall distribution of main subtypes was somehow comparable among patients with liver disease, matched controls and blood donors. The predominance of subtypes 4k, 4r and 4q was found in the three groups (Figure 6).

When distribution of the subtypes was considered in patients with liver disease alone, subtypes 4r, 4a and 4b combined were more likely to be isolated from patients with advanced liver disease than from cases with non-advanced forms. They were present in 41.9% among patients with LC and/or HCC versus 17.8% in patients with recent or chronic hepatitis respectively (OR=3.33[1.26-8.83], p=0.016]). This could have been linked to advanced age with HCV infection by those strains for those with advanced liver disease but a strong association persisted when we controlled for age (OR=2.96[0.98-8.93], p=0.055]). Moreover, we found in the phylogenetic tree that four strains in a sub-clade of 6 HCV subtype 4r were from cases with advanced liver disease (Table 2 & Figure 3, paper III). If these strains could be more virulent than other subtypes prevalent in Rwanda is subject to further studies. To our knowledge, none has evaluated specific virulence of different subtypes for HCV genotype 4 to serve as reference in explaining the present findings.

In contrast to HBV strains in Rwanda, the predominance and high diversity of HCV genotype 4 strains in Rwanda may indicate that HCV has been present in Rwanda for a relatively long time and the different subtypes have spread all over

the country (Figure 6). A hypothesis on Central African origin of HCV genotype 4 has been raised previously, from where strains may have been spread across the whole region, to the North of the continent and to the Middle East in the period of early 20th century through invasive procedures, especially vaccination campaigns[359,376-378]. The distribution of the HCV genotype 4 strains isolated from Rwanda presented in this paper III and paper II shows a large spread of the four major subtypes across the country (figure 6).

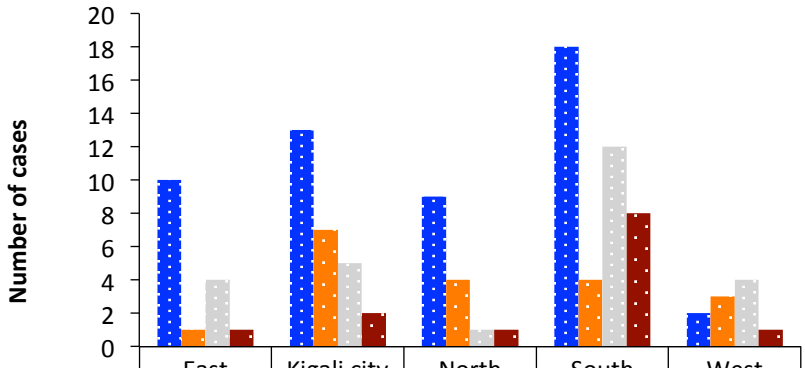
The predominance of subtypes 4k and 4r is shared with the Democratic Republic of Congo but HCV subtypes 4r strains have also been isolated in more distant countries like Ethiopia and Saudi Arabia but were not found in Burundi[358,359,379,380]. Therefore, there might be multiple sources of HCV strains that are prevailing in Rwanda as well. All the thirteen (11%) of the HCV genotype 4 strains that could not be classified in any of existing class of subtypes were also spread into different clades to widen further the variability of these HCV strains isolated from Rwanda.

4.3.6. HBV genotypes and subtypes circulating in liver disease patients

Of all strains from 49 samples with detectable HBV DNA, 26 could be sequenced. Twenty-three were clustering in a same clade within the subgenotype A1 as the 53 of the 58 HBV strains from blood donors described in **paper I.** This raised at 92.9% the rates of Rwandan subgenotype A1 strains forming that clade (figure 2, paper III).

This limited variability of isolated HBV strains in Rwanda can be linked to a possible recent introduction of this HBV subgenotype A1 strain in the country, which has spread all of over the country without any newly introduced strains as previously stated. Unfortunately, the exclusive prevalence of HBV subgenotype

A1 and their low variability could not allow any evaluation of impact of genotypes on liver disease progression. However mutations in pre-S and BCP regions need to be evaluated in that regard to investigate any potential association of one or combined mutations and the advanced forms of HBV-induced liver disease.



	East	Kigali city	North	South	West
Subtype 4k	10	13	9	18	2
Subtype 4q	1	7	4	4	3
Subtype 4r	4	5	1	12	4
Subtype 4	1	2	1	8	1

Figure 6. Histogram illustrating the distribution of predominant HCV genotype 4 subtypes (4k, 4q, 4r and untyped 4) isolated from blood donors, patients with liver disease and controls in Rwanda according provinces of origin of samples

4.4. PAPER IV: Prevalence of markers for enteric hepatitis viruses in Rwanda and their current epidemiological patterns

In this study we performed analyses on samples obtained from blood donors used in studies for papers I and II and samples collected from liver disease patients and matched controls used in the study for paper III. In total, 1,212 study participants were enrolled in this study. The median age was 33 years (interquartile range: 25; 45 years). Females represented 31% while almost 49% were farmers and 84% were living in the four rural provinces of Rwanda. Of the 1,212 eligible samples, 1,045 could be analyzed for anti-HAV antibodies and 1,133 samples for anti-HEV IgG. All 126 samples positive for anti-HEV IgG and 65 with borderline titers were subject to anti-HEV IgM assay and real-time PCR for HEV RNA detection (figure 1 & table 3 of paper IV).

4.4.1. Seroprevalence of anti-HAV and associated factors

The overall seroprevalence for anti-HAV in these study participants was 96.9% and the rates were increasing with age. The seroprevalence was lower in participants younger than 25 years (94%) compared with the older group (98%, p=0.001). However, no significant difference of seroprevalence was found between farmers and participants with other occupations after adjustment for age. Likewise, there was no difference with regard to province of origin, as between Kigali city and the rural provinces (p=0.562) (table 1, paper IV).

Incidence of acute hepatitis A remains important among children in the SSA region and high seroprevalence among adults are somehow expected in this endemic region including Rwanda. However, relatively lower prevalence of anti-HAV indicating a lack of HAV protective immunity found in some young adult participants has not been reported before in the East African region [17,381]. This could be linked to a possible recently arising decrease of exposure to HAV among children in Rwanda probably due to improved socioeconomic conditions and hygiene. In case that decline is sustained, an increasing number of adults

without anti-HAV antibodies could be expected. As the risk to contract HAV infection in that country remains important, those who may acquire HAV infection during their adulthood will bear a risk to develop severe and life-threatening acute fulminant hepatitis A, relatively common for HAV infections contracted in late adulthood[19,20,382-384]. Therefore, there is a need to continue with regular surveillance and to introduce the HAV vaccine in Rwanda for individuals at risk to prevent them from the possible severe acute fulminant episodes.

4.4.2. Prevalence and factors for current or past HEV infection markers

Of 1,133 samples analyzed for anti-HEV IgG, 126 were positive (11.1%) and 65 (5.7%) had borderline anti-HEV IgG titers. Only eight of 125 (6.4%) positive samples for IgG and five from those with borderline IgG (7.7%) had anti-HEV IgM, a marker of recent HEV infection (figure 1, paper IV). Three samples with anti-HEV IgG including one with anti-HEV IgM and four samples with borderline anti-HEV IgG titers, all without anti-HEV IgM, were HEV RNA positive. In summary, we had 135 samples (11.9%) with at least one positive marker of HEV current or past infection: 126 samples with anti-HEV IgG, five samples with anti-HEV IgM while with borderline anti-HEV IgG and four samples with HEV RNA without positive reactivity for anti-HEV IgG or IgM.

This prevalence of markers for current or past HEV infection of 11.9% in Rwanda was somehow comparable to that reported from some European countries previously considered as non-endemic and from countries of Southeast Asian region[385-387]. It was relatively lower than the prevalence reported from other African countries[388-391]. However, different reasons may keep this confirmation tempered. Firstly, existing serological assays for HEV are discordant and there is no gold standard yet to calibrate those assays. The seroprevalence in this study should have been overestimated at 16.9% if we

have used the cut point of 1 S/CO recommended by the manufacturer. This hinders an objective comparison of seroprevalence between studies when different serological assays or different cut-off points have been used[296,306,392]. Secondly, in most countries with available data on anti-HEV seroprevalence including SSA countries, this generally increased with age and age-differences between participants may impact on their comparability[245,388,389,393].

Interestingly, we did not find any age-dependent increasing prevalence of HEV markers in this study. The prevalence somehow peaked at 17.7% in the age group of 36-45 years but remained comparable across all age groups (table 3, paper IV). In regression analysis, age was not found as an independent factor for presence of HEV markers when adjusted for gender, occupation and residence (adj. OR [95%CI]=1.00[0.98-1.02], p=0.836; table 3, paper IV). This could indicate that HEV, at least predominant prevalent strains, have been recently introduced in the country and almost all adult ages have had the same risk of exposure.

Finally, the prevalence of HEV markers also varied with factors predisposing to HEV infection in different regions. These factors include, but are not limited to, a poorer access to clean water, high density of pigs, culinary and diet habits of eating raw pork meat or liver sausage and occupations exposing to zoonotic transmission of some specific HEV strains dominated by HEV3 to date[245,394-397].

4.4.3. High probability of HEV3 strains' presence in Rwanda

In this study, we observed a regional difference for prevalence of HEV markers. Western and Southern provinces accounted for the highest prevalence of 18.6% and 17.1 % respectively. The lowest prevalence was found in Eastern province (5.5%) while it was 5.9% in Kigali city (figure 2, paper IV). The two provinces with the highest prevalence also account for the highest densities of pigs in Rwanda whereas this density is lowest in Eastern province, which is rather

known for the highest density of cattles[398]. Thus, there might be a predominance of HEV strains transmitted from swine to humans in Rwanda.

Since the SSA region is known for different outbreaks of HEV1 and HEV 2 strains that are endemic in the region, the lack of age-dependent pattern and the hypothesis of newly introduced strains could refer to a possible presence of HEV3 strains brought from endemic countries for HEV3 to Rwanda with the importation of pigs[23,239,240,399]. Cases of HEV3 strains closely related to strains infecting swine have been repeatedly isolated in developed countries[400,401] and epidemiology of HEV3 infection has been linked to consumption of raw or not well cooked pig meat and/or liver sausages[402].

Recently, HEV3 strains have also been found in samples from patients with liver disease in South Africa and from pigs in Democratic Republic of Congo and Cameroun but hypothesis of their introduction in those countries with imported pigs from Europe could not be completely elucidated [276,391,403,404] In Rwanda, pig breeds from Europe are common, as they have been imported since more than a decade to improve the national livestock. It is estimated that imported races represent up to 60% of pig breeds in the country[405]. Unfortunately we could not succeed to sequence any of the strains isolated from the 7 samples with detected HEV RNA. This can be due to low viral loads as the Ct values were ranging between 30.4 and 41.5 or to a possible prevalence of divergent strains in Rwanda that could not be amplified with the primers used. This deserves additional works to identify those circulating strains and compare them to those that may be circulating in pigs.

4.4.4. Profile of HEV infection in Rwanda and potential consequences

Of seven HEV RNA positive samples, four were from repeat blood donors who presumably did not present any symptom and who were negative for other markers screened for blood donation in Rwanda namely HIV, syphilis, HBsAg,

and anti-HCV. Blood products from these donors may have probably been used to patients. Recipients may incur a risk to contract HEV infection that may take a chronic course well described for HEV3 especially if patients are immune deficient[227,272]. On the other hand, the transfusion-transmitted HEV infection can cause an acute hepatitis that is generally self-limiting. However, life-threatening acute hepatitis has been reported in SSA region and Asia for HEV1 strains in pregnant women[23,240]. Severe forms of acute hepatitis due to HEV3 infections have also been described but with a low case fatality rate in pregnant women from developed countries[266]. Moreover, acute HEV infection on preexisting chronic liver disease can cause decompensation of liver disease with an associated high mortality [25,268,269,406]. Therefore, results from this paper unveil for the first time in Africa an additional transfusion safety of HEV transmission, which also deserves special considerations particularly when ordering blood products for patients in those categories at high risk.

Apart from infected blood donors, three samples from patients diagnosed with liver disease were also HEV RNA positive. All presented with nonspecific signs and symptoms such as a mild fever, jaundice, fatigue, abdominal pain and hepatomegaly and moderately elevated liver enzymes[27,255]. None of could be diagnosed in Rwanda probably due to lack of awareness of HEV. Then, the diagnosis of HEV infection needs to be emphasized on when caring patients with acute hepatitis in particular.

CONCLUDING REMARKS

Seroprevalence of HBsAg among Rwandan blood donors in 2014 was intermediate while that of anti-HCV was rather low in young adults but increases with age. Anti-HCV prevalence might be overestimated especially in blood donors by up to 67% due to high rates of false positive when only Elisa-based assays are used for screening. There was no evidence of active or past HDV infection in Rwanda. Seroprevalence of anti-HAV was expectedly high but relatively lower in younger generations due to a recent and ongoing decline of exposure during childhood. Prevalence of HEV markers among Rwandan adults was moderate with geographical discrepancies and highest prevalence in areas with high densities of pigs breeding.

Hepatitis viruses have a predominant implication in liver disease in Rwanda and were found responsible of about ¾ of cases of liver disease. HBV and HCV were foremost the primary drivers with HCV being particularly more important in liver cirrhosis and somehow in hepatocellular carcinoma. Both HBV and HCV were modestly diagnosed in acute hepatitis but the role of HEV was far under-recognized despite its presence.

Epidemiology of HBV in Rwanda showed predominance in young males, a potential sexual and intra-familial transmission. The latter is shared with HCV but in contrast, HCV showed an age-dependent increasing prevalence and is more likely to be diagnosed in females older than 49 years probably exposed many years back to unsafe healthcare practices at home or at health facilities during pregnancies and/or deliveries. The HEV epidemiological pattern in Rwanda features a zoonotic mode of transmission from pigs but further works are needed to be explored this.

Circulating HBV strains are exclusively of genotype A, subgenotype A1 sharing a same genetic origin and with limited variability. HCV strains were mainly of genotype 4 but with various subtypes spread across the country. Subtypes 4k, 4r, 4q were dominant and an important proportion of untyped HCV genotype 4 strains were identified. HCV subtypes 4r, 4b and 4a seemed to be associated with advanced forms of liver disease indicating a possible higher virulence but this needs also to be further explored.

FUTURE PERSPECTIVES

Although we could hypothesize that the majority of current patients with advanced liver disease may have been infected during previous decades, HCV and HBV routes of transmission for relatively new cases need to be explored in a specific epidemiological study to have results that may provide an orientation to preventive efforts in order to expect to attain the goal of eradication of these viruses in Rwanda.

In the same line, clinical researches should be performed on patients undergoing treatment for HCV and identify potential mechanisms of failure that may arise.

For HBV, contribution of different mutants and variants on the progression of chronic hepatitis B to cirrhosis and HCC deserve specific further works as well with a special emphasize on identification of precore and BCP mutants and their impact on clinical outcome.

For enterically transmitted viruses, there is a need to continue a regular evaluation of anti-HAV seroprevalence that may allow a timely recognition of need for introduction of the HAV vaccine to prevent severe acute hepatitis A in adults without immunity.

An epidemiological study for HEV is also needed with a particular focus on identification of circulating strains among HEV-infected humans and pigs and their relationship. In the same line with HEV, a policy for prevention of transfusion-transmitted HEV infections to vulnerable groups is also needed in Rwanda since there is no screening for HEV in the country as in many other countries in the world.

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